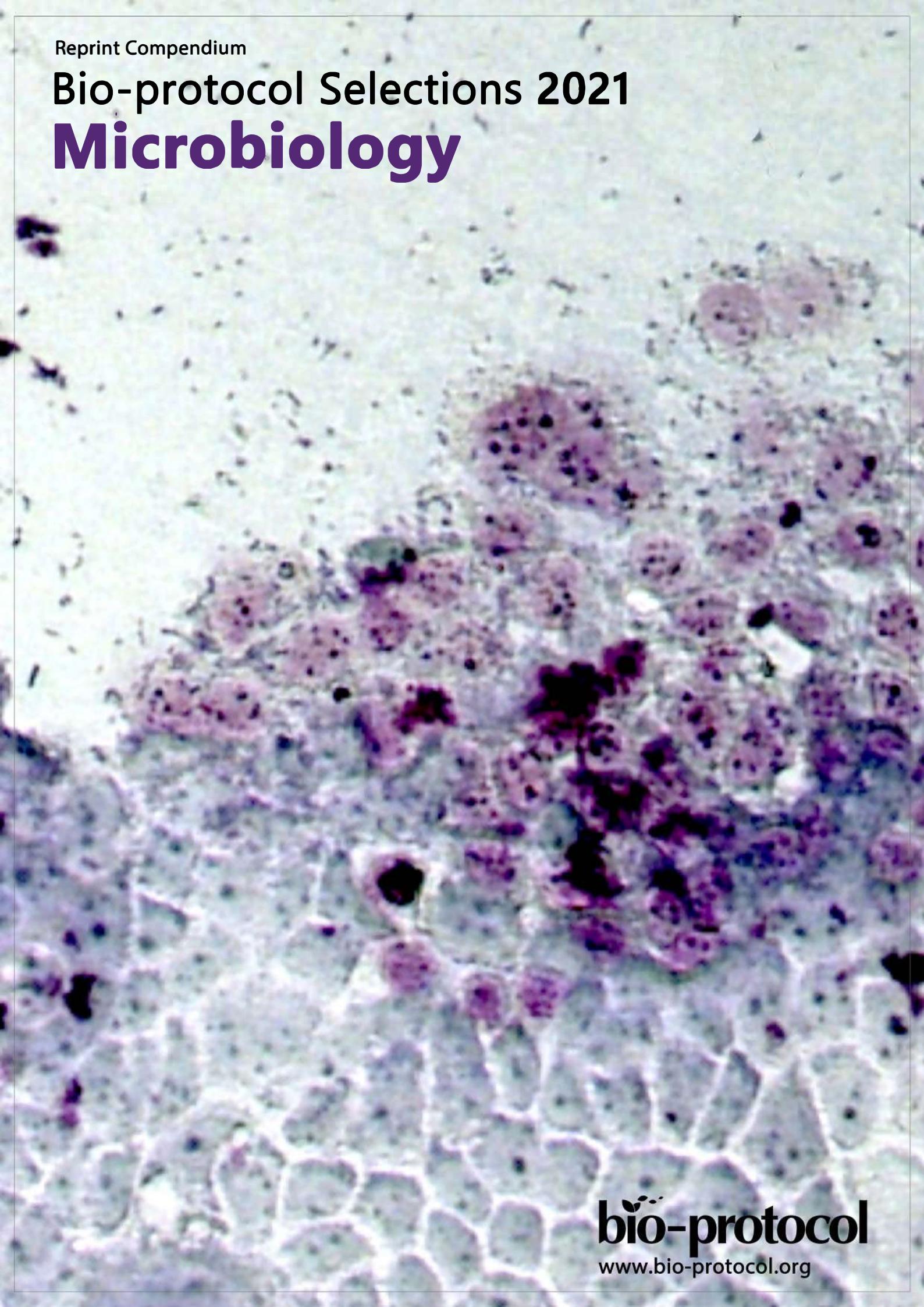


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1. Isolation of Thylakoid Membranes from the Cyanobacterium *Synechocystis* sp. PCC 6803 and Analysis of Their Photosynthetic Pigment-protein Complexes by Clear Native-PAGE 1
Komenda *et al.* 【Original Research Article: Biochim Biophys Acta Bioenerg 858(5),337-350】
2. Assessing Yeast Cell Survival Following Hydrogen Peroxide Exposure 11
Tran *et al.* 【Original Research Article: J Biol Chem 293(37), 14429-14443】
3. Quantification of Hepatitis B Virus Covalently Closed Circular DNA in Infected Cell Culture Models by Quantitative PCR 22
Qu, B. and Urban, S. 【Original Research Article: J Virol 92(23), e01117-18】
4. Analysis of Indole-3-acetic Acid (IAA) Production in Klebsiella by LC-MS/MS and the Salkowski Method 35
Gang *et al.* 【Original Research Article: Plant Soil 424, 273–288】
5. Enterovirus Competition Assay to Assess Replication Fitness 44
Lulla, V. and Firth, A. E. 【Original Research Article: Nat Microbiol 4(2), 280-292】
6. TZA, a Sensitive Reporter Cell-based Assay to Accurately and Rapidly Quantify Inducible, Replication-competent Latent HIV-1 from Resting CD4+ T Cells 51
Sanyal *et al.* 【Original Research Article: Nat Med 23(7), 885-889】
7. An Improved Bioassay to Study *Arabidopsis* Induced Systemic Resistance (ISR) Against Bacterial Pathogens and Insect Pests 65
Cecchini *et al.* 【Original Research Article: Mol Plant Microbe Interact 32(1), 86-94】
8. Biofilm Formation Assay in *Pseudomonas syringae* 78
Shao *et al.* 【Original Research Article: Mol Plant Microbe Interact 31(12),1232-1243】
9. Fluorescence Microscopy Assay to Measure HIV-1 Capsid Uncoating Kinetics *in vitro* 89
Chantal *et al.* 【Original Research Article: Elife, e34772】

-
10. Plaque Assay to Determine Invasion and Intercellular Dissemination of *Shigella flexneri* in TC7 Human Intestinal Epithelial Cells 112
Sharma, A. and Puhar, A. 【Original Research Article: Immunity 39(6), 1121-31】
 11. QUEEN-based Spatiotemporal ATP Imaging in Budding and Fission Yeast 124
Masak Takaine 【Original Research Article: J Cell Sci 132(8), jcs230649】
 12. Yeast Single-cell RNA-seq, Cell by Cell and Step by Step 140
Nadal-Ribelles *et al.* 【Original Research Article: Nat Microbiol 4(4), 683-692】
 13. Production of Quantum Dots-containing Influenza Virus Particles for Studying Viral Uncoating Processes 162
Qin *et al.* 【Original Research Article: Proc Natl Acad Sci U S A 116(7), 2577-2582】
 14. Quantification of HIV-2 DNA in Whole Blood 177
Szojka *et al.* 【Original Research Article: Lancet HIV S2352-3018(18)30254-6】
 15. Localizing Genome Segments and Protein Products of a Multipartite Virus in Host Plant Cells... 197
Vernerey *et al.* 【Original Research Article: Elife, e43599】
 16. Herpes Simplex Virus Type 1 Propagation, Titration and Single-step Growth Curves 211
Grosche *et al.* 【Original Research Article: J Cell Biol 218(2), 508-523】
 17. Protocol for Ribosome Profiling in Bacteria 234
Mohammad, F. and Buskirk, A. R. 【Original Research Article: J Cell Biol 218(2), 508-523】
 18. Sulfatase Assay to Determine Influence of Plants on Microbial Activity in Soil 256
Koprivova *et al.* 【Original Research Article: Proc Natl Acad Sci U S A 116(31), 15735-15744】
 19. Transcervical Mouse Infections with *Chlamydia trachomatis* and Determination of Bacterial Burden 265
Rajeeve, K. and Sivadasan, R. 【Original Research Article: Nat Microbiol 3(7), 824-835】

-
20. Contemporaneous Measurement of Outer and Inner Membrane Permeability in Gram-negative Bacteria.....276
Ma *et al.* 【Original Research Article: *Nat Commun* 10(1), 3517】
21. Quantification of Bacteria Residing in *Caenorhabditis elegans* Intestine.....283
Palominos, M. and Calixto, A. 【Original Research Article: *Nat Commun* 10(1), 3517】
22. HIV-CRISPR: A CRISPR/Cas9 Screening Method to Identify Genes Affecting HIV Replication 295
Roesch, F. and OhAinle, M. 【Original Research Article: *Elife*, e39823】
23. Screening Method for CRISPR/Cas9 Inhibition of a Human DNA Virus: Herpes Simplex Virus....315
Neuhäusser *et al.* 【Original Research Article: *Elife*, e51662】
24. An *in vitro* DNA Sensor-based Assay to Measure Receptor-specific Adhesion Forces of Eukaryotic Cells and Pathogens337
Wack *et al.* 【Original Research Article: *Nat Commun* 11(1), 32】
25. *Candida albicans* Culture, Cell Harvesting, and Total RNA Extraction.....358
Cravener, M. V. and Mitchell, A. P. 【Original Research Article: *PLoS Genet* 16(1), e1008582】
26. Colorimetric RT-LAMP Methods to Detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)366
Park *et al.* 【Original Research Article: *J Mol Diagn* 22(6), 729-735】
27. Rapid Isolation and Purification of Secreted Bacteriocins from *Streptococcus mutans* and Other Lactic Acid Bacteria.....377
Cheng *et al.* 【Original Research Article: *J Bacteriol* 202(12), e00762-19】
28. Generating Three-dimensional Human Granulomas *in vitro* to Study *Mycobacterium tuberculosis*-host Interaction389
Arbués *et al.* 【Original Research Article: *PLoS Pathog* 16(2), e1008312】

-
29. A Quick Method for Screening Biocontrol Efficacy of Bacterial Isolates Against Bacterial Wilt Pathogen *Ralstonia solanacearum* in Tomato397
Agarwal *et al.* 【Original Research Article: Microbiological Research 238: 126503】
30. Long-distance Transport in Bacterial Swarms Revealed by Single Nanoparticle Tracking408
Feng, J. and He, Y. 【Original Research Article: iScience 22, 123-132】
31. A Mismatch-tolerant RT-LAMP Method for Molecular Diagnosis of Highly Variable Viruses420
Li *et al.* 【Original Research Article: Front Microbiol 10, 1056】
32. I Plate-based Assay for Studying How Fungal Volatile Compounds (VCs) Affect Plant Growth and Development and the Identification of VCs via SPME-GC-MS432
Wang *et al.* 【Original Research Article: Mol Plant Microbe Interact 31(10), 1021-1031】

On the Cover:



Image from the protocol “**Plaque Assay to Determine Invasion and Intercellular Dissemination of *Shigella flexneri* in TC7 Human Intestinal Epithelial Cells**”. *Bio-protocol* 9(13): e3293. DOI: 10.21769/BioProtoc.3293.

Isolation of Thylakoid Membranes from the Cyanobacterium *Synechocystis* sp. PCC 6803 and Analysis of Their Photosynthetic Pigment-protein Complexes by Clear Native-PAGE

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[Abstract] Cyanobacteria represent a frequently used model organism for the study of oxygenic photosynthesis. They belong to prokaryotic microorganisms but their photosynthetic apparatus is quite similar to that found in algal and plant chloroplasts. The key players in light reactions of photosynthesis are Photosystem I and Photosystem II complexes (PSI and PSII, resp.), large membrane complexes of proteins, pigments and other cofactors embedded in specialized photosynthetic membranes named thylakoids. For the study of these complexes a mild method for the isolation of the thylakoids, their subsequent solubilization and analysis is essential. The presented protocol describes such a method which utilizes breaking the cyanobacterial cells using glass beads in an optimized buffer. This is followed by their solubilization using dodecyl-maltoside and analysis using optimized clear-native gel electrophoresis which preserves the native oligomerization state of both complexes and allows the estimation of their content.

Keywords: Cyanobacteria, Photosynthesis, Thylakoids, Photosystem, Clear-native gel, Electrophoresis

[Background] Thylakoids are specialized membranes of algal and plant chloroplasts and cyanobacteria. The thylakoid lipid bilayer shares characteristic features with prokaryotic bacterial membranes but it is enriched in glycolipids, namely galactolipids, while the level of acidic lipids is relatively low and does not exceed 20% of the overall lipid content. Thylakoids contain a large number of proteins and their complexes and over 40% of them are involved in light-dependent reactions of photosynthesis. The next largest functional group includes proteins involved in protein targeting, processing and folding, oxidative stress response and translation. The most integral membrane proteins of thylakoids form large protein complexes which play an important role in the harvesting of light and its photochemical conversion. The key complexes performing this conversion are PSI and PSII. They both perform the light-driven primary charge separation resulting in the thylakoid-associated flow of electrons reducing NADP⁺ and generating a pH gradient used for the synthesis of ATP. Both complexes are subject to intensive research focused mainly on the elucidation of their function and underlying structural features as well as the mechanism of their assembly. For this research the isolation of native membranes and their detailed analysis as concerns the content, assembly and oligomerization state of both photosystems is essential. The existing and frequently used protocols for the isolation of cyanobacterial thylakoids either do not sufficiently preserve the native structure of fragile membrane complexes like Photosystem II dimers (e.g.,

Komenda and Barber, 1995), or the obtained preparations contain high concentrations of compounds (for instance 1 M glycine betaine) which are not compatible with the clear native gel electrophoresis (*e.g.*, Gombos *et al.*, 1994). The described protocol for thylakoid preparation is optimized from both points of view. The analysis of cyanobacterial membranes by clear native PAGE (CN PAGE) using a protocol modified from Wittig *et al.* (2007) gives direct information about the quantity and oligomeric (or assembly) state of multisubunit pigmented photosystem complexes with unprecedented speed and resolution. The isolation, solubilization, and analysis of membranes does not disrupt weak non-covalent interactions between pigments and proteins and therefore the gel with separated individual complexes can be directly scanned. Unlike denaturing SDS gels, no fixation, staining and destaining, or, blotting and immunodetection is needed for distinguishing between PSII and PSI (see Figure 2). Green PSI complexes do not show red fluorescence at room temperature and are present as dominant green bands in the gel while PSII complexes fluoresce and their green bands are hard to distinguish on the simple transparency scan. Thus, PSI quantification is done using the simple transparency scan of green non-fluorescent bands while PSII quantification is done using the red fluorescence scan of fluorescent bands.

Materials and Reagents

1. Pipette tips
2. 2 ml screw cap polypropylene micro vials (Biospec Products, catalog number: 3205)
3. Hamilton® microsyringe series 700, 0.25 ml (Sigma, catalog number: 24538-U)
4. Hamilton needle, 51 mm (Sigma, catalog number: 21746)
5. Binder Clip (Deli 8584, Luma trading)
6. Magnetic stirring bars with diameter 5 mm and length 20 mm
7. Transparent plastic film
8. 1.5 ml spectroscopic cuvettes (Fisherbrand PS, catalog number: 7755.0302)
9. The glucose tolerant strain of the cyanobacterium *Synechocystis* sp. PCC6803 (GT-P, Tichý *et al.*, 2016)
10. Acrylamide for molecular biology (AA) (AppliChem Panreac, catalog number: A3812,1000)
11. Bisacrylamide for molecular biology (BIS) (AppliChem Panreac, catalog number: A3636,0250)
12. 6-aminohexanoic acid (ACA) (AppliChem Panreac, catalog number: A2266,500)
13. Bis-Tris (AppliChem Panreac, catalog number: A1025.1000)
14. MES (AppliChem Panreac, catalog number: A0689.0250)
15. Tricine (AppliChem Panreac, catalog number: A1085,1000)
16. N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, catalog number: T928-25ml)
17. Ammonium persulfate (APS) (Sigma, catalog number: T3678-25g)
18. Dithiothreitol (DTT) (AppliChem Panreac, catalog number: A1101,0025)
19. SDS (Sigma, catalog number: 7172-100g)
20. Glass beads with a diameter of 150-212 µm (Sigma, catalog number: G1145)
21. n-Dodecyl-β-D-maltoside (DM) (AppliChem Panreac, catalog number: A0819,0005)

22. Sodium deoxycholate (Sigma, catalog number: 30970-25g)
23. Gel Filtration Markers Kit for Protein Molecular Weights (Sigma, catalog number: MWGF1000-1KT)
24. CaCl₂ (analytical grade)
25. MgCl₂ (analytical grade)
26. Methanol (analytical grade)
27. Sodium hydroxide (analytical grade)
28. Hydrochloric acid (analytical grade)
29. Glycerol (HPLC grade)
30. Milli-Q de-ionized water (Merck Millipore, Ultrapure [Type 1] water)
31. Buffer B (see Recipes)
32. Gel buffer 6x (pH 7.6) (see Recipes)
33. Acrylamide solution (AB) (see Recipes)
34. Upper (Cathode) buffer 10x (see Recipes)
35. Upper (Cathode) buffer 1x (see Recipes)
36. Lower (Anode) buffer 10x (see Recipes)
37. Lower (Anode) buffer 1x (see Recipes)
38. Resolving gel and stacking gel (see Recipes)

Equipment

1. Automatic pipettes (P1000, P200 and P10)
2. Mini-beadbeater (Sigma, catalog number: Z249289)
3. Magnetic stirrer Color Squid White (Thermo Fisher Scientific, catalog number: 6110.1005)
4. GE gradient maker (Sigma, catalog number: GESG50)
5. Vortex (Velp Scientifica, model: RX3)
6. Centrifuge (Hettich, model: Micro22 R)
7. UV-VIS spectrophotometer (Shimadzu, model: UV3000)
8. Cooled microcentrifuge (Eppendorf, model: 5415R)
9. PAGE apparatus (Bio-Rad, PROTEAN® II xi Cell)
10. Power Supply (Bio-Rad, PowerPac™ HV High-Voltage)
11. Cooled thermostat (Labio, model: CTB 06C)
12. LAS 4000 camera (Fuji; for chlorophyll fluorescence scanning used LED blue light source 460 nm for excitation and R670 filter for emission)
13. Scanner HP ScanJet G4050 (HP, model: G4050)
14. pH meter (Sartorius, PB-11)
15. FIM-150 flake ice maker (Biobase)

Software

1. ImageJ bundled with Java 1.8.0_112 (<https://imagej.nih.gov/ij/download.html>)

Procedure

1. The protocol requires 20 ml of *Synechocystis* PCC 6803 grown at 30 °C on an orbital shaker (about 60 rpm) under cool white fluorescent light of 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in liquid BG11 (Rippka *et al.*, 1979) with an optical density of ~0.4-0.6 at 750 nm (see Notes). Collect the cyanobacterial cells by centrifugation at 2,300 $\times g$ for 10 min at ~4 °C, resuspend the cell pellet in 1.5 ml of Buffer B and transfer the resuspended cells into a 2 ml screw cap microtube. From this point keep the tubes on ice.
2. Centrifuge the tube in the microcentrifuge at 9,300 $\times g$ for 2 min at 4 °C, discard the supernatant and resuspend the pellet in 0.15 ml of Buffer B and add the same volume of glass beads (measured separately in a graduated 0.5 ml microtube and added to the 2 ml microtube with the sample).
3. Break the cells with three cycles of 20 s beating (max. speed) and 1 min cooling in a Mini-Beadbeater.
4. Add 0.2 ml of Buffer B, vortex the tube for 2 s and immediately transfer the supernatant into a new pre-cooled 2 ml screw cap microtube, repeat five times.
5. Centrifuge the tube at 400 $\times g$ for 1 min at 4 °C to remove unbroken cells and glass beads.
6. Transfer the supernatant into a new pre-cooled 2 ml screw cap microvial and centrifuge at 16,000 $\times g$ for 20 min at 4 °C.
7. Remove supernatant and resuspend the pellet in 0.2 ml of Buffer B using a stainless-steel piston from a 0.25 ml microsyringe (length 120 mm, diameter 2 mm): hold the tube with the pellet after removal of the supernatant in one hand and piston in the second hand, insert the piston into the tube down to the bottom and vortex for 15 s with inserted piston, then add 0.2 ml of the buffer and vortex again with piston inside for 10 s.
8. Measure the chlorophyll (Chl) content in the suspension. Mix 13.5 μl of suspension with 1.5 μl of 10% DM, centrifuge at 16,000 $\times g$ for 5 min, take 10 μl of the supernatant and add to 990 μl of methanol in a screw cap microvial, vortex, centrifuge at 16,000 $\times g$ for 5 min, take the supernatant and measure absorbance at 720 and 666 nm, C ($\mu\text{g Chl/ml}$) = $(A_{666} - A_{720}) \times 1,269$ (Wellburn, 1994).
9. Prepare the resolving gel (see Video 1).
 - a. First assemble the gel cassettes from glass plates and 1 mm spacer according to the manufacturer's instructions.
 - b. Then mix all components well (except for APS!) for each 4% and 14% resolving gel solution using magnetic stirrers and stirring bars (components of each solution are shown in Recipe 8, one column is one solution, the second and third column); then add APS to both solutions under constant mild stirring.

- c. Pour the 4% solution together with stirring bar into the right compartment of the gradient maker located on the top of the magnetic stirrer. The gradient maker has an inlet attached to the silicone tube leading to the peristaltic pump and has a closed connecting channel to the second compartment. The outlet of the peristaltic pump is connected to the hypodermic needle attached to the upper part of the glass gel cassette using a binder clip.
- d. Carefully open the connecting channel just enough to fill it with the 4% solution (if more solution is released, pipette it back to the right compartment) and close it again, and add now the 14% solution into the second (left) compartment of the gradient maker.
- e. Remove 8.5 ml of the 14% solution with a pipette and add it directly to the bottom of the glass cassette for gel polymerization and immediately start pumping the remaining solution with a rate of about 10 ml per minute into the glass cassette and in parallel open the connecting channel between the compartments of the maker. Adjust mild stirring of the solution (2 turns per second).
- f. After pouring the gradient, remove the needle, overlay the solution in the gel cassette with 0.5 ml of distilled water using a 0.25 ml syringe and wash the gradient maker and tubings with Milli-Q water. Watch the interface between the water and 4% solution until a clear border is formed and then remove the water with the syringe.



Video 1. Pouring the resolving gel

10. Prepare the stacking gel. Add APS to the 10 ml 4% solution (right column in Recipe 8), pour to the top of the polymerized resolving gel, immerse the 10 well comb avoiding air bubbles and wait for polymerization; afterwards remove the comb and wash the wells with Milli-Q water.
11. Attach the prepared gels into the cooling core, check if the connection is tight and not leaking with distilled water, pour out water and insert the core with gels into the electrophoresis chamber with 2 litres of 1x lower buffer, attach the silicon tubes of the cooling core to the thermostat, which is set to 4 °C and pour 200 ml of 1x upper buffer into the upper reservoir of the cooling core.

12. For gel analysis take the samples containing 5 µg Chl, add a corresponding volume of Buffer B buffer to reach the same volume for all samples (usually 30 µl), add 1/10 of the volume of 10% DM and centrifuge immediately at 16,000 x g for 20 min at 4 °C.
13. Take 25 µl of the solubilized membranes and load into the gel wells.
14. For estimation of the molecular size of complexes, Gel Filtration Markers Kit for Protein Molecular Weights can be used, load 30 µl of the recommended mixture.
15. Close the lid, attach it to the power supply and run in the constant current mode at 15 mA for 1.5-2 h with an upper voltage limit of 1,000 V (see Figure 1; during the run voltage gradually increases up to 1,000 V, after reaching this limit, the current goes down).



Figure 1. The setup of the electrophoretic apparatus (lower right corner) with cooling thermostat (left) and power supply (upper right corner)

16. After the pigmented front reaches a distance of about 8 cm from the bottom of the wells, switch off the power supply, remove the lid, detach the silicone cooling tubes and take out the cooling core with the gels from the electrophoretic chamber.
17. Remove the gel from the glass cassette and wrap it into a transparent plastic film.
18. Transfer it into the scanner to acquire the color image (300 dpi resolution, 48-bit color depth).
19. Transfer the gel into the LAS4000 camera to take a 16 bit greyscale image in tiff format either in transparency mode to quantify green bands of PSI (Digitize DIA method, the lowest tray position, 3 s exposure, high resolution mode) or in epifluorescence mode using the blue LED excitation light (wavelength 460 nm) eliciting the chlorophyll (but not phycobiliprotein) fluorescence while the emitted light is passed through an R670 filter (long pass filter with the edge at 670 nm) to quantify PSII (Fluorescence method, the lowest tray position, 3 s exposure, high-resolution mode) (see Figure 2).

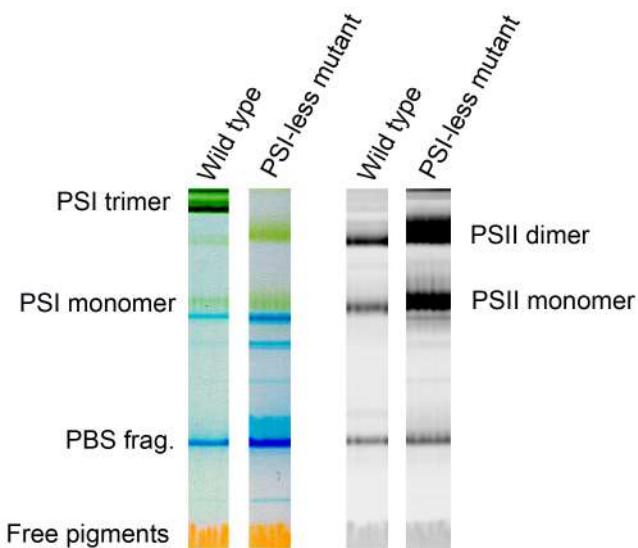


Figure 2. Representative color scans (left pair) and chlorophyll fluorescence images (right pair) of the gel with separated Photosystem I and Photosystem II complexes from the cyanobacterium *Synechocystis* PCC 6803 GT-P (Tichý *et al.*, 2016) and the Photosystem I-less mutant (Shen *et al.*, 1993). Left two lanes show color scans, right ones Chl fluorescence scans with dominant bands of Photosystem II dimer and monomer. Blue bands (PBS frag.) represent fragments of phycobilisomes, the cyanobacterial antennae, fluorescence of which is not elicited by blue light.

20. Quantify bands using ImageJ according to the user guide (<https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>). The bands are first selected as regions of interest (ROIs) using the Area selection tools in Toolbar Tools, the background is subtracted using the processing tools and the intensity of the bands analyzed using the intensity statistics in the analysis tools.

Notes

Apart from the cyanobacterium *Synechocystis* PCC 6803 the described protocol has already been successfully tested for other cyanobacterial strains such as *Synechococcus elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002. For the green algae *Chlamydomonas reinhardtii*, *Chlorella sorokiniana* and *Scenedesmus quadricauda* the protocol is identical, only larger glass beads with a diameter of 0.5 mm (Sigma) should be used for breaking the cells. The procedure for CN PAGE can also be used for isolated thylakoid membranes from plant chloroplasts.

Recipes

1. Buffer B (50 ml stock)

25 mM MES/NaOH buffer (pH 6.5)

10 mM CaCl₂

10 mM MgCl₂

25% glycerol

Divide into 10 ml aliquots and store frozen at -25 °C (stable for at least 6 months)

2. Gel buffer 6x (pH 7.0, 100 ml)

300 mM Bis-Tris/HCl buffer

Adjust to pH 7.0 with 4 M HCl

Store at 4 °C (stable for at least 6 months at 4 °C)

Note: Usually 100 ml is prepared.

3. Acrylamide solution (AB)

50% AA

1.33% BIS

Milli-Q water

Store at 4 °C (stable for at least 3 months)

Note: Usually 500 ml is prepared.

4. Upper (Cathode) buffer 10x

0.5 M tricine

150 mM Bis-Tris/HCl

Adjust pH to 7.0 using 4 M HCl

Store at 4 °C (stable for at least 6 months at 4 °C)

Note: Usually 500 ml is prepared.

5. Upper (Cathode) buffer 1x (200 ml for one PAGE)

a. Add 20 ml of upper buffer 10x to 180 ml of Milli-Q water and 1 ml of 10% sodium deoxycholate to reach 0.05% final concentration

b. Add 0.4 ml of 10% DM to reach 0.02% final concentration.

Note: Prepare fresh before running.

6. Lower (Anode) buffer 10x

0.5 M Bis-Tris/HCl

Adjust pH to 7.0 with 4 M HCl

Store at 4 °C (stable for at least 6 months at 4 °C)

Note: Usually 500 ml is prepared.

7. Lower (Anode) buffer 1x (2 L)

Dilute lower (Anode) buffer 10x to lower (Anode) buffer 1x with Milli-Q water

Note: This solution is stable for at least 3 months at 4 °C and can be reused three times.

8. Resolving gel and stacking gel

Mix individual components (the first column from left) to get 4% (second column) and 14% (third column) solution for the preparation of the resolving gradient gel and 4% solution for the preparation of the stacking gel (fourth column) according to the following table:

	4% (resolving gel)	14% (resolving gel)	4% (stacking gel)
Glycerol	0	3.2 g	0 g
Water	6.5 ml	6.1 ml	7.4 ml
Buffer	1.4 ml	2.8 ml	1.7 ml
AB	0.68 ml	4.8 ml	0.8 ml
10% DM	43 µl	86 µl	50 µl
TEMED	3.5 µl	7.0 µl	10 µl
10% APS	19 µl	38 µl	80 µl
Volume	8.5 ml	17 ml	10 ml

Acknowledgments

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Competing interests

We declare no conflict of interests.

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Assessing Yeast Cell Survival Following Hydrogen Peroxide Exposure

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[Abstract] In the presence of oxidative stress, cellular defense systems that can detoxify reactive oxygen species are activated through multiple signaling cascades and transcriptional reprogramming. The budding yeast *Saccharomyces cerevisiae* has served as an excellent model for genetically-identifying factors important for the response to oxidative stress. Here, we describe two assays for testing yeast gene deletion strains or strains overexpressing a gene of interest for viability following oxidative stress induced by hydrogen peroxide treatment. These include a plate-based spot assay for visualizing cell growth and a quantitative colony counting assay. As stress response assays can be highly variable depending on cell growth conditions, these protocols have been optimized for obtaining highly-reproducible results between experiments. We demonstrate the use of these protocols for genetic tests of a putative chromatin regulator implicated in regulating the transcriptional response to oxidative stress.

Keywords: Yeast, Oxidative stress, Stress responses, Hydrogen peroxide, Cell survival, Yeast overexpression, Yeast mutants, Spot assays, Colony forming units

[Background] The unicellular eukaryote *Saccharomyces cerevisiae* has been an exceptional model for revealing large networks of genes critical to the survival of cells in diverse types of environmental stress (Gasch *et al.*, 2000; Weiner *et al.*, 2012; Ho and Gasch, 2015), largely due to the excellent genetic tools available in this system and the ease with which stress responses can be evaluated. In particular, genetic studies in yeast have uncovered the regulatory mechanisms that control the cells' response to oxidative stress (Morano *et al.*, 2012). Environmental factors, including radiation and the presence of redox-cycling agents, are a source of oxidative stress which cause increased reactive oxygen species (ROS). These ROS include the superoxide anion, H₂O₂, and hydroxyl radicals, which are highly reactive with proteins, lipids, and nucleic acids, causing oxidative damage to all of these macromolecules (Schieber and Chandel, 2014). Aerobic respiration also generates low levels of endogenous ROS by the leakage of electrons from the electron transport chain, and mitochondrial dysfunction, as occurs with stress or aging, increases ROS levels further, which triggers the induction of cellular defense mechanisms such as activated expression of antioxidant enzymes which detoxify ROS (Schieber and Chandel, 2014). Pathologies including cancer, cardiovascular disease, autoimmune diseases, and aging are associated with deregulated oxidative stress defense systems and altered levels of intracellular ROS. Therefore, identifying and characterizing the network of proteins responsible for protecting cells during oxidative stress is key to defining the pathophysiology underlying these diseases.

To test the role of a given gene in the response to oxidative stress, it is advantageous to analyze yeast

strains which are deleted or otherwise mutated for the gene and which overexpress the protein encoded by the gene. If a particular gene is required to protect cells during oxidative stress, it is expected that its deletion would render cells sensitive to the stress, and its overexpression would cause resistance to the stress. The assays described below test the survival of yeast cells following treatment with hydrogen peroxide, which has served as a model compound for inducing oxidative stress in yeast (Morano *et al.*, 2012; Kwolek-Mirek and Zadrag-Tecza, 2014). Specifically, these protocols were used to test the role of the chromatin regulator Set4 during oxidative stress (Tran *et al.*, 2018). We used a *set4Δ* yeast strain, and a strain carrying a plasmid for the inducible overexpression of *SET4* in the presence of β-estradiol, as previously described (McIsaac *et al.*, 2013). The raw data shown here were obtained to generate the results published in Tran *et al.* (2018) that demonstrate a role for Set4 in protecting cells during oxidative stress.

We present both a semi-quantitative plate spot assay and a quantitative colony forming unit (cfu) survival assay to interrogate the survival of cells following hydrogen peroxide treatment. We found it useful to be able to visualize the growth differences in cells on the plates and validate these differences quantitatively using the cfu assay. Both protocols here appeared to show much more consistent results than plate spot assays in which hydrogen peroxide is added to the medium in the plate prior to spotting the yeast on the plate (Kwolek-Mirek and Zadrag-Tecza, 2014). In addition, we applied methods to optimize the growth conditions and hydrogen peroxide concentration to account for the observations that yeast cells acquire resistance to hydrogen peroxide when grown in nutrient-poor media, such as synthetic complete or dropout media, or following the diauxic shift, which occurs when cells transition from primarily glucose metabolism via glycolysis to aerobic respiration of ethanol (Guan *et al.*, 2012). When these conditions are not accounted for, results are highly variable and may represent confounding factors in the experiment, rather than the consequences of the deletion or overexpression of the gene being investigated. These assays are not resource-intensive and can be used to test multiple mutant or overexpressing strains simultaneously. Overall, these protocols represent a sensitive and highly-reproducible approach to assaying the response of yeast cells to oxidative stress.

Materials and Reagents

1. Pipette tips
 - a. P2 tips (Gilson, catalog number: F171200)
 - b. P200 tips (Gilson, catalog number: F171300)
 - c. P1000 tips (Gilson, catalog number: F171500)
2. Toothpicks and inoculating sticks (sterilized) (VWR, catalog number: 12000-806)
3. Petri dish (Corning, catalog number: 351029)
4. Assay Plate, 96-well, U bottom (Falcon, catalog number: 62406-015)
5. 1.5 ml microfuge tubes (Fisherbrand, catalog number: 05-408-129)
6. 50 ml conical centrifuge tubes (Falcon, catalog number: 14-432-22)

7. Yeast strains (Table 1)
 - a. yEG001
 - b. yEG322
 - c. yEG315
 - d. yEG372
 - e. yEG375
8. Vector pMN3 (empty vector) (McIsaac *et al.*, 2013)
9. Vector pMN3-*SET4* (Tran *et al.*, 2018)
10. Sterile, distilled water
11. Yeast extract (Research Products International, catalog number: Y20025)
12. Peptone (Research Products International, catalog number: P20250)
13. Dextrose (Thermo Scientific, catalog number: BP350-1)
14. Yeast nitrogen base (US Biologicals, catalog number: Y2025)
15. Drop-out Mix Synthetic Minus Uracil (US Biologicals, catalog number: D9535)
16. Ethanol (Sigma-Aldrich, catalog number: E7023)
17. Hydrogen peroxide (Fisher Scientific, catalog number: H325-100)
18. β-estradiol (Millipore Sigma, catalog number: E2758-1G)
19. Agar (US biological, catalog number: A0930)
20. YPD (Yeast extract-Peptone-Dextrose) medium (see Recipes)
21. Synthetic Complete without Uracil (SC-URA) medium (see Recipes)
22. 10 mM β-estradiol (see Recipes)

Table 1. Yeast strains used in this protocol

Strain	Background	Genotype	Ref.
yEG001	BY4741	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	YKO
yEG322	BY4741	<i>MATα set4Δ::HIS3MX</i>	Tran <i>et al.</i> (2018)
yEG315	DBY12394	<i>MATα ura3Δ leu2Δ0::ACT1pr-Z3EV-NATMX</i>	McIsaac <i>et al.</i> (2013)
yEG372	DBY12394	<i>MATα ura3Δ leu2Δ0::ACT1pr-Z3EV-NATMX + p183 (pMN3)</i>	McIsaac <i>et al.</i> (2013)
yEG375	DBY12394	<i>MATα ura3Δ leu2Δ0::ACT1pr-Z3EV-NATMX + p180 (pMN3-SET4)</i>	Tran <i>et al.</i> (2018)

Equipment

1. 125 ml (or 50 ml, if available) Erlenmeyer flasks (VWR, catalog number: 10536-912)
2. Glass cell spreaders (VWR, catalog number: 89166-788)
3. Single-channel pipettors

- a. P2 pipette (Gilson, catalog number: F144054M)
- b. P20 pipette (Gilson, catalog number: F144056M)
- c. P200 pipette (Gilson, catalog number: F144058M)
- d. P1000 pipette (Gilson, catalog number: F144059M)
4. Glass culture tubes (15 ml) (Corning, catalog number: 982016X)
5. Multichannel Pipettors (Pipetman Neo P8 x 20N and Pipetman Neo P8 x 200N)
6. Incubator
7. Rollordrum Tc-7 Tissue Culture Rotator (New Brunswick Scientific)
8. Incubator Shaker (New Brunswick Scientific G-25, temperature required: 30 °C; shaker dimensions: 22 in. x 45.5 in. x 30.5 in)
9. Vortexer (VWR Standard Heavy Duty Vortex Mixer, catalog number: 97043-562)
10. Spectrophotometer (Thermo Scientific, model: NanoDrop 2000c, catalog number: ND-2000c)
11. MultiDoc-it Imaging system (UVP)
12. Centrifuge (Beckman Coulter Allegra X-14R, catalog number: A99465)
13. Microfuge (Beckman Coulter Microfuge 20R)
14. Autoclave (Steris AMSCO Century SG-120 Scientific Gravity Sterilizer)

Software

1. Excel, Microsoft Office
2. Prism GraphPad

Procedure

- A. Assessing survival of wild-type and mutant yeast cells by spot assay
 1. Streak wild-type and mutant yeast strains (Table 1) to single colonies on YPD plates. Incubate for 2 days at 30 °C.
 2. Using a sterile toothpick, select individual wild-type, and mutant colonies. Resuspend the colony in 100 µl YPD in a centrifuge tube. Measure the OD₆₀₀ using a UV-VIS spectrophotometer (e.g., NanoDrop 2000c) and calculate to determine the volume required to achieve a final cell concentration of OD₆₀₀ equals 0.0006 in 30 ml YPD. Dilute this volume of culture into 30 ml YPD and grow overnight (~16-18 h for a colony from a plate stored at 4 °C, or ~12-14 h for a colony from a plate coming from the 30 °C incubator) to mid-log phase (OD₆₀₀ ~0.4-0.5) at 30 °C with shaking at 220 rpm.
 3. Measure the OD₆₀₀ of the cultures. Determine the volume of culture equivalent to ~12 OD₆₀₀ units. For example, 12 OD₆₀₀ units of a culture with an OD₆₀₀ of 0.5 equals 24 ml culture (divide 12 by 0.5). Transfer the calculated volume to a 50 ml conical centrifuge tube and centrifuge at 3,900 x g for 3 min.
 4. Remove supernatant, and resuspend cells in 20 ml pre-warmed YPD.

5. Mix cultures well by vortexing, and add 10 ml of culture to two sterile flasks for each strain being tested.
6. To one of the flasks, add H₂O₂ to 4 mM final concentration in 10 ml culture. Commercially-available H₂O₂ is usually 30% (w/w), density of 1.1 g/ml, and has a molecular weight of 34.01 g/mol. Therefore, the stock concentration is 9.79 M, and 4.08 µl of the stock gives a final concentration of 4 mM in a 10 ml culture volume. The calculation for determining the molarity of the stock solution is shown below:
 - a. Stock solution: 30 g of H₂O₂ per 100 g H₂O
 - b. 100 g H₂O/1.1 g/ml = 90.9 ml of solution for 30 g H₂O₂
 - c. (30 g H₂O₂/90.9 ml) × 100 = 33.3 g H₂O₂ in 100 ml H₂O
 - d. 33.3 g H₂O₂/34.01 g/mol = 0.979 mol in 100 ml = 9.79 M
7. Place all flasks in the shaker and continue incubating at 30 °C for 30 min.
8. After the 30 min incubation, remove 1 ml from each culture and place in a 1.5 ml microfuge tube. Centrifuge at 12,200 × g for 2 min.
9. Aspirate supernatant and resuspend the cell pellet in 1 ml sterile, distilled water.
10. Set up a 10-fold dilution series of cells in a 96-well plate. You will have a separate row of cells for each strain. In the first well, add 200 µl of undiluted cells. In the next five wells, add 180 µl of sterile water. For each strain, take 20 µl of the undiluted cells and add it to the next well (containing 180 µl of water) using a multichannel pipette. Pipet up and down to mix sample. Then take 20 µl of this dilution and add it to the next well. Pipet up and down to mix and transfer 20 µl to the next well. Repeat to finish the dilution series.
11. Using a multichannel pipette, spot 5 µl from each well onto a YPD plate. Start with the most dilute samples and end with the undiluted samples.
12. Let the spots dry on the plates, and then transfer to the incubator at 30 °C.
13. Cells from untreated cultures should be visible by day 2, whereas cells from cultures treated with H₂O₂ may take up to 5 days to be visible. Take pictures of the plates every day on a black background with white light using a gel documentation or other imaging system. Representative images are shown in Figure 1. A flow-chart outlining the steps in this protocol is provided in Figure 2.

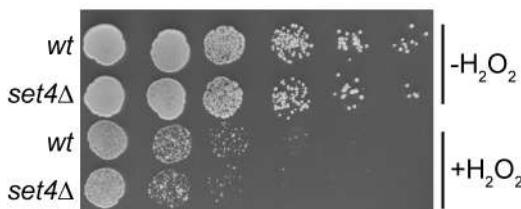


Figure 1. Representative image of a spot assay YPD plate showing serial dilutions of *wt* and *set4Δ* cells either untreated or treated with 4 mM H₂O₂ prior to spotting on the plate.
Image was acquired after two days incubation at 30 °C. This type of data corresponds to the experimental outline described in Procedure A.

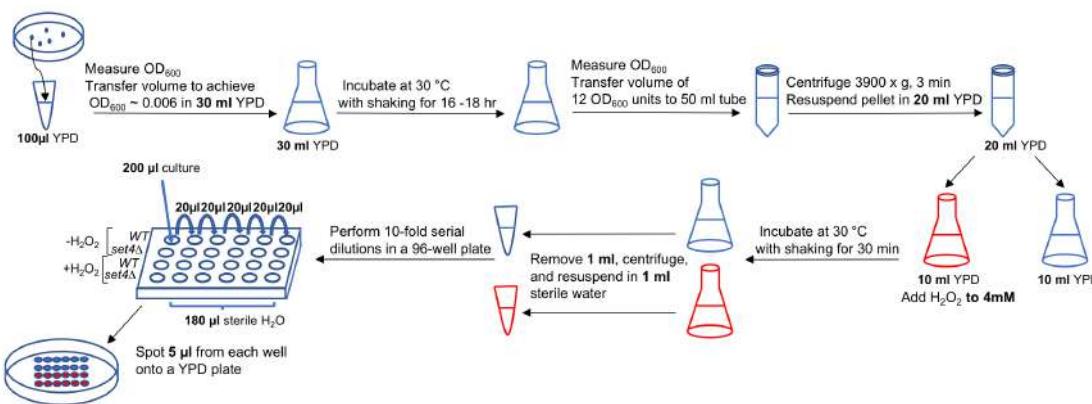


Figure 2. Flow-chart describing experimental outline for Procedure A. Many of the steps shown in the flow-chart are similar to those used in Procedure C, in which *SET4* (or a gene of interest) is overexpressed.

B. Assessing survival of wild-type and mutant yeast cells by counting colony forming units (cfu)

1. Follow Steps A1-A2 to grow yeast strains, except grow only 10 ml of each culture instead of 30 ml.
2. When the cultures have reached mid-log phase (OD₆₀₀ ~0.4-0.5), remove 10 μl of each culture to perform serial dilutions, and follow Step B3 to treat the remaining culture with H₂O₂. While the culture is incubating (as described below), dilute the 10 μl into 990 μl sterile, distilled water, and then perform a second 1:100 serial dilution. Plate 100 μl of the diluted cells onto a YPD plate using a glass plate spreader, representing a 1:1 × 10⁵ dilution (or a dilution factor, DF, of 10⁵) of the original culture.
3. While performing dilutions and plating cells, treat the cultures with H₂O₂ to 4 mM final concentration (4.08 μl of the H₂O₂ stock in the 10 ml culture volume). Continue shaking at 30 °C for 30 min.
4. After the incubation, remove 10 μl of each culture and dilute once into 990 μl sterile, distilled water. Plate 100 μl of the diluted cells onto a YPD plate using a glass plate spreader, representing a 1:1 × 10³ dilution (or a dilution factor, DF, of 10³) of the original culture.
5. Incubate the plates at 30 °C for 2 days. Count colonies on each of the plates and record values to calculate percent survival, as shown in Table 2 and described in the Data Analysis section. A flow-chart outlining the steps in this protocol is shown in Figure 3.

Table 2. Raw data and percent survival calculations for assessing survival of yeast overexpressing *SET4*

	Replicate	<i>Empty vector (yEG372)</i>	<i>SET4 vector (yEG375)</i>	
Colony counts		before H ₂ O ₂	after H ₂ O ₂	before H ₂ O ₂
	1	70	218	54
	2	80	89	41
	3	99	129	39
Total cells		DF: 10 ⁵	DF: 10 ³	DF: 10 ⁵
	1	7,000,000	218,000	5,400,000
	2	8,000,000	89,000	4,100,000
	3	9,900,000	129,000	3,900,000
Percent survival	1		3.11	
	2		1.11	
	3		1.30	
	Mean	1.84		7.17
	SEM	0.55		0.77

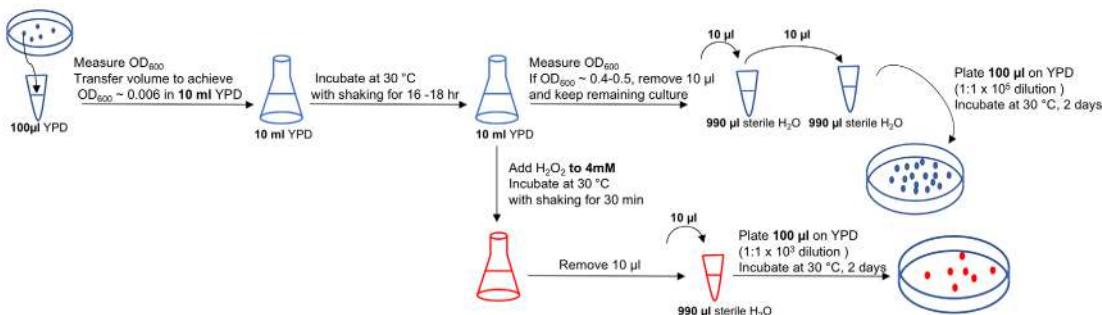


Figure 3. Flow-chart describing experimental outline for Procedure B. Many of the steps shown in the flow-chart are similar to those used in Procedure D, in which *SET4* (or a gene of interest) is overexpressed.

C. Assessing survival of yeast overexpression strains by spot assay

- For testing survival of cells overexpressing a gene of interest, yeast strain yEG315 was used and transformed (Gietz and Schiestl, 2007) with the vector pMN3, which drives overexpression of a cloned gene in the presence of β-estradiol (strains and plasmids provided by Scott McIsaac; Table 1 [McIsaac *et al.*, 2013]).
- Streak yeast containing the empty vector or overexpression vector onto SC-URA plates to single colonies. Incubate for 2 days at 30 °C.
- Pick a single colony using a sterile inoculating stick and inoculate 3 ml of SC-URA medium in a 15 ml glass culture tube and grow overnight with shaking at 30 °C.
- The next day, measure the OD₆₀₀ using a spectrophotometer. Dilute the cultures to OD₆₀₀ ~0.1-0.2 in 20 ml SC-URA medium.

5. Freshly prepare 10 mM β -estradiol in ethanol. Further dilute to 0.1 mM in ethanol and add 1 μ M β -estradiol to each culture.
 6. Incubate the cultures with shaking at 30 °C. Grow to mid-log phase (OD_{600} ~0.5-0.7; approximately 4-6 h).
 7. Measure the OD_{600} of the cultures and calculate the volume of culture equivalent to ~12 OD_{600} units, as described in Procedure A.
 8. Transfer the calculated volume to a 50 ml conical centrifuge tube and centrifuge at 3,900 $\times g$ for 3 min.
 9. Resuspend in 20 ml pre-warmed SC-URA medium.
 10. Swirl to mix cultures well, and remove 10 ml of culture to a new flask so that you have two flasks for each strain being tested.
 11. To one of the flasks, add H_2O_2 to 20 mM final concentration (20.43 μ l of the H_2O_2 stock in the 10 ml culture volume).
 12. Place all flasks in the shaker and continue incubating at 30 °C for 30 min.
 13. After the 30 min incubation, remove 1 ml from each culture and place in a 1.5 ml microfuge tube. Centrifuge at 12,200 $\times g$ for 2 min.
 14. Aspirate supernatant and resuspend the cell pellet in 1 ml sterile, distilled water.
 15. Follow Steps A10-A13 to set up 10-fold serial dilutions, spot cells on SC-URA plates, and take images of the plates after 2-3 days of growth at 30 °C.
- D. Assessing survival of yeast overexpression strains by counting colony forming units (cfu)
1. Follow Steps C1-C4, except grow only 10 ml cultures in SC-URA (rather than 20 ml).
 2. Freshly prepare 10 mM β -estradiol in ethanol. Further dilute to 0.1 mM in ethanol and add 50 nM β -estradiol to each culture.
 3. When the cultures have reached mid-log phase (4-6 h at 30 °C; OD_{600} ~0.5-0.7), remove 10 μ l of each culture and perform serial dilutions. Dilute the 10 μ l into 990 μ l sterile, distilled water, and perform a second 1:100 serial dilution. Plate 100 μ l of the second dilution onto an SC-URA plate using a glass plate spreader, representing a 1:1 $\times 10^5$ dilution of the original culture.
 4. While performing dilutions and plating cells, treat the cultures with a final concentration of 20 mM H_2O_2 (20.43 μ l of the H_2O_2 stock in the 10 ml culture volume) for 30 min. Continue shaking at 30 °C.
 5. After the incubation, remove 10 μ l of each culture and dilute once into 990 μ l sterile, distilled water. Plate 100 μ l of the diluted cells onto an SC-URA plate using a glass plate spreader, representing a 1:1 $\times 10^3$ dilution of the original culture.
 6. Incubate the plates at 30 °C for 2 days. Count colonies on each of the plates and record values to calculate percent survival, as shown in Table 2.

Data analysis

For spot assay tests, plates were imaged using a gel documentation system to qualitatively assess survival following hydrogen peroxide treatment. Examples of these data are shown in Figures 2A and 2E in Tran *et al.* (2018). To quantitatively measure survival using the colony forming unit (cfu) assay, the total number of colonies on each plate was multiplied by the dilution factor (DF). Percent survival for each replicate was determined by dividing the total colony counts of the H₂O₂-treated cells by the total colony counts of the untreated cells and multiplying by 100, as outlined in Table 2. The average of the replicates was determined, and the statistical significance was evaluated using an unpaired *t*-test. These data were analyzed with GraphPad Prism. The raw data and calculations shown in Table 2 are represented graphically in Figure 2F in Tran *et al.* (2018).

Notes

For both sets of protocols described here, the recommended culture volume to grow to log-phase and perform the H₂O₂ treatment is a minimum of 10 ml in an Erlenmeyer flask. Although this is a much larger volume than is required for either the spot assay or the cfu assay, we found this volume of culture growth to provide more reproducible results. When similar assays were performed with cells grown in culture tubes and smaller volumes of medium, results were more variable, possibly due to decreased aeration of the cultures.

Another consideration for the H₂O₂ treatment steps is the different media types used here, either rich YPD medium or synthetic medium. Cells grown in synthetic medium (complete or dropout) develop inherent stress resistance and therefore exhibit higher survival rates upon H₂O₂ treatment. Therefore, a higher concentration of H₂O₂ is required when cells are grown in synthetic medium (such as in Procedures C and D), rather than rich medium. We found that 4 mM H₂O₂ in YPD and 20 mM H₂O₂ in synthetic medium produced comparable survival rates in wild type cells. However, depending on precise media composition and growth conditions, testing a range of H₂O₂ concentrations in a pilot experiment may be useful for assay optimization.

Recipes

1. YPD (Yeast extract-Peptone-Dextrose) medium (for 1 L)

10 g yeast extract

20 g peptone

20 g dextrose (glucose)

Add distilled water to 1 L, mix well, and autoclave to sterilize

To make YPD plates, add 25 g agar prior to autoclaving. Pour into Petri plates after autoclaved material has cooled enough to be handled

2. Synthetic Complete without Uracil (SC-URA) medium (for 1 L)
1.92 g uracil drop-out mix
6.7 g yeast nitrogen base
20 g dextrose (glucose)
Add distilled water to 1 L, mix well, and autoclave to sterilize
To make SC-URA plates, add 25 g agar prior to autoclaving. Pour into Petri plates after autoclaved material has cooled enough to be handled
3. 10 mM β-estradiol
1.1 mg β-estradiol
400 µl ethanol
Make fresh just before use. Dilute further as described in protocol if necessary for accurate pipetting

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Quantification of Hepatitis B Virus Covalently Closed Circular DNA in Infected Cell Culture Models by Quantitative PCR

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[Abstract] Persistence of the human hepatitis B virus (HBV) requires the maintenance of covalently closed circular (ccc)DNA, the episomal genome reservoir in nuclei of infected hepatocytes. cccDNA elimination is a major aim in future curative therapies currently under development. In cell culture based *in vitro* studies, both hybridization- and amplification-based assays are currently used for cccDNA quantification. Southern blot, the current gold standard, is time-consuming and not practical for a large number of samples. PCR-based methods show limited specificity when excessive HBV replicative intermediates are present. We have recently developed a real-time quantitative PCR protocol, in which total cellular DNA plus all forms of viral DNA are extracted by silica column. Subsequent incubation with T5 exonuclease efficiently removes cellular DNA and all non-cccDNA forms of viral DNA while cccDNA remains intact and can reliably be quantified by PCR. This method has been used for measuring kinetics of cccDNA accumulation in several *in vitro* infection models and the effect of antivirals on cccDNA. It allowed detection of cccDNA in non-human cells (primary macaque and swine hepatocytes, etc.) reconstituted with the HBV receptor, human sodium taurocholate cotransporting polypeptide (NTCP). Here we present a detailed protocol of this method, including a work flowchart, schematic diagram and illustrations on how to calculate “cccDNA copies per (infected) cell”.

Keywords: Hepatitis B Virus, Covalently closed circular DNA, cccDNA, T5 exonuclease, Quantitative PCR, Copy number per cell

[Background] The Hepatitis B virus (HBV), a DNA virus belonging to the family *Hepadnaviridae*, is a human pathogen persisting in approximately 240 million people globally. HBV infection leads to higher risks of liver cirrhosis and hepatocellular carcinoma (Liang *et al.*, 2015). At present, chronic HBV infection is not curable as current treatments do not eradicate the replicative reservoir, covalently closed circular (ccc)DNA (Levrero *et al.*, 2016). In *in vitro* infected hepatocytes, cccDNA is formed by cellular repair of relaxed circular duplex (rc)DNA, the genomic form in virions (Guo *et al.*, 2007; Long *et al.*, 2017; Schreiner and Nassal, 2017). cccDNA serves as the viral template for pregenomic RNA which becomes encapsidated into nucleocapsids and is reverse transcribed to generate rcDNA and double-stranded linear (ds)DNA by the viral polymerase (Bartenschlager and Schaller, 1992; Tu *et al.*, 2017). Of note, albeit the reconstitution of hepatoma cells with the receptor human sodium taurocholate cotransporting polypeptide (NTCP), high excess of inoculated virions with high multiple genome equivalents (mges)

(mges > 100) is required to achieve moderate infection rates (e.g., > 20% in HepG2^{NTCP} cells) (Qu *et al.*, 2018). This raises the problem that cccDNA-containing samples taken early after inoculation with HBV contain large amounts of rcDNA from input virions.

Therefore, adequate methodologies for absolute or relative quantification of cccDNA are required. They are divided into two categories: (I) hybridization after separation by gel electrophoresis (Southern blot) and (II) PCR amplification (Li *et al.*, 2017). Southern blot is still a gold standard but not very sensitive, and demands multiple experimental processes and a high-copy load of cccDNA (> 2 × 10⁶ copies using ³²P-radioactive isotope/digoxigenin/biotin probe; > 1 × 10⁴ copies using branched DNA technique) (Yu *et al.*, 2015). Therefore, hybridization-based methods are complicated, time-consuming and not practical for a large number of samples (e.g., > 20) to be analyzed in parallel. PCR methods include real-time quantitative (q)PCR, nested qPCR (Xu *et al.*, 2011), digital-droplet PCR (Mu *et al.*, 2015), and rolling circle amplification (Margeridon *et al.*, 2008). Real-time qPCR is the fastest and the most robust method for almost all laboratories. However, unlike Southern blot which gel electrophoretically separates cccDNA, PCR methods are not strictly specific, especially when rcDNA and other HBV replicative intermediates are present in excess, such as in *in vitro* infection (e.g., MGE > 300), even when cccDNA-specific primer pairs are used (Nassal, 2015; Qu *et al.*, 2018).

To solve this problem, we have developed a qPCR assay using validated cccDNA selective primer pairs and a digestion step by T5 exonuclease, which removes cellular DNA and all HBV intermediates via its exonuclease activity targeting free ends of rcDNA and dsIDNA but leaves cccDNA intact (Qu *et al.*, 2018). This assay allowed fast and specific quantification of cccDNA within one working day as shown in Figure 1 (2 h of total DNA extraction; 1.5 h of T5 exonuclease reaction and 2 h of qPCR), accurate calculation of “cccDNA copies/(infected) cell” (Figure 2) and drug efficacy testing on cccDNA levels (Figure 3). The method also provided quantitative judgment on whether cccDNA is formed in new cell models and identified low amount of cccDNA in *in vitro* infection of non-primate hepatocytes (Lempp *et al.*, 2016 and 2017). This protocol is adapted from Qu *et al.* (2018) and herein more detailed information on this qPCR quantification after T5 exonuclease digestion is included, and different primer pairs are compared to address the applicability of HBV genotypes. Taken together, this protocol will facilitate studies on cccDNA and help clinicians, technicians and graduate students to analyze cccDNA in samples derived from *in vitro* infection.

Materials and Reagents

1. Pipette tips (Neptune, 1,000 µl, 200 µl, 20 µl, 10 µl, DNase-/RNase-free & Biozym, premium tips 1000 µl, 200 µl, 10 µl)
2. 1.5 ml microcentrifuge tube (Sarstedt AG & Co.KG, SafeSeal tube, catalog number: 72.706)
3. 0.2 ml microcentrifuge tube (Greiner Bio-one, Sapphire PCR tube, catalog number: 683271)
4. Hard-Shell PCR plates 96-well, thin-wall (Bio-rad, catalog number: HSP9601)
5. Primary human hepatocytes (PHH) (obtained from Hannover medical school or prepared in University Hospital Heidelberg) (Possible commercial vendors of PHH are Lonza, Biopredic,

BioLV, ThermoFisher, however susceptibility to HBV may vary), HepaRG^{NTCP} and HepG2^{NTCP} cell lines (Qu *et al.*, 2018)

6. Plasmid pSHH2.1 (Cattaneo *et al.*, 1983), available upon request from the corresponding author

7. Primers:

Primer p1040: 5'-GTGGTTATCCTGCGTTGAT-3'

Primer p1996: 5'-GAGCTGAGGCGGTATCT-3'

p1578: 5'-CCGTGTGCACTCGCTTCA-3'

p1867: 5'-GCACAGCTTGGAGGCTTGA-3'

p1583: 5'-TGCACTTCGCTTCACCT-3'

p2301: 5'-AGGGGCATTGGTGGTC-3'

8. Probe

Probe p1085: 5'-FAM-AGTTGGCGAGAAAGTGAAGCCTGC-TAMRA-3'

9. Trypsin [0.25% Trypsin-EDTA (1x)] (Invitrogen, Gibco, catalog number: 25200-056), 4 °C

10. NucleoSpin Tissue kit (Macherey-Nagel, catalog number: 740952.250)

11. Buffer T1 (Macherey-Nagel, catalog number: 740940.25)

12. Proteinase K (Macherey-Nagel, catalog number: 740506), -20 °C

13. Buffer B3 (Macherey-Nagel, catalog number: 740920)

14. Ethanol absolute (VWR chemicals, catalog number: 20821.330)

15. Buffer BW (Macherey-Nagel, catalog number: 740922)

16. Buffer B5 Concentrate (Macherey-Nagel, catalog number: 740921)

17. T5 exonuclease (New England Biolabs, catalog number: M0363), -20 °C

18. NEBuffer 4 (New England Biolabs, catalog number: B7004), -20 °C in aliquots

19. PerfeCTa qPCR Toughmix (Quanta Biosciences, catalog number: 95112-012), -20 °C in aliquots

20. SYBR Green Supermix (Bio-Rad, catalog number: 172-5121), -20 °C in aliquots

21. Myrcludex B (Bachem), available upon request from the corresponding author, -80 °C in aliquots

22. Recombinant human Interferon-α-2a (PeproTech, catalog number: 300-02AA), -80 °C in aliquots

23. Human Interferon-α-2a (PBL Assay Science, catalog number: 11100-1), -80 °C in aliquots

24. Nuclease-free water (B. Braun Melsungen, Aqua ad iniecatilia Braun)

25. NaCl (Carl Roth GmbH, catalog number: 9265.2)

26. KCl (Sigma-Aldrich, catalog number: 31248)

27. Na₂HPO₄·2H₂O (Sigma-Aldrich, catalog number: 04272)

28. KH₂PO₄ (Sigma-Aldrich, catalog number: P9791)

29. Tris-base (Carl Roth GmbH, catalog number: 4855.2)

30. Dimethyl sulfoxide (Sigma-Aldrich, catalog number: 1.02950)

31. Fetal bovine serum gold (PAA Laboratories GmbH, catalog number: A15-151), -20 °C

32. Penicillin/Streptomycin (Thermo Fisher Scientific, catalog number: 15140-122), 4 °C

33. L-glutamine (Thermo Fisher Scientific, catalog number: 25030-024), 4 °C

34. MEM non-essential amino acids (Thermo Fisher Scientific, catalog number: 11140-035), 4 °C

35. Recombinant insulin (Sigma-Aldrich, catalog number: 91077C-1G), -20 °C in aliquots
36. Hydrocortisone 21-hemisuccinate sodium salt (Sigma-Aldrich, catalog number: H4881), -20 °C in aliquots
37. Culture medium (see Recipes)
38. 10x PBS buffer (see Recipes)
39. 5 mM Tris-HCl (see Recipes)

Equipment

1. Pipettes (Gilson P1000, P200, P20; Eppendorf P2.5)
2. Cell counter (Bio-Rad, TC20™ Automated Cell Counter, catalog number: 1450102)
3. Thermomixer (Eppendorf, ThermoMixer C, catalog number: 5382000015)
4. Microcentrifuge (Thermo Fisher Scientific, Heraeus™ Pico™ 21 Centrifuge, catalog number: 75002553)
5. Thermocycler (Labrepco, Biometra T3000 Thermocycler 48, catalog number: 050-723)
6. qPCR thermocycler (Bio-Rad, C1000 Touch™ Thermal cycler with 96-well Fast Reaction Module, catalog number: 1851196)

Software

1. CFX96 Real-time System
(Bio-Rad, <http://www.bio-rad.com/en-us/product/cfx96-touch-real-time-pcr-detection-system>)
2. CFX Manager™ Software
(Bio-Rad, <http://www.bio-rad.com/en-us/sku/1845000-cfx-manager-software?ID=1845000>)

Procedure

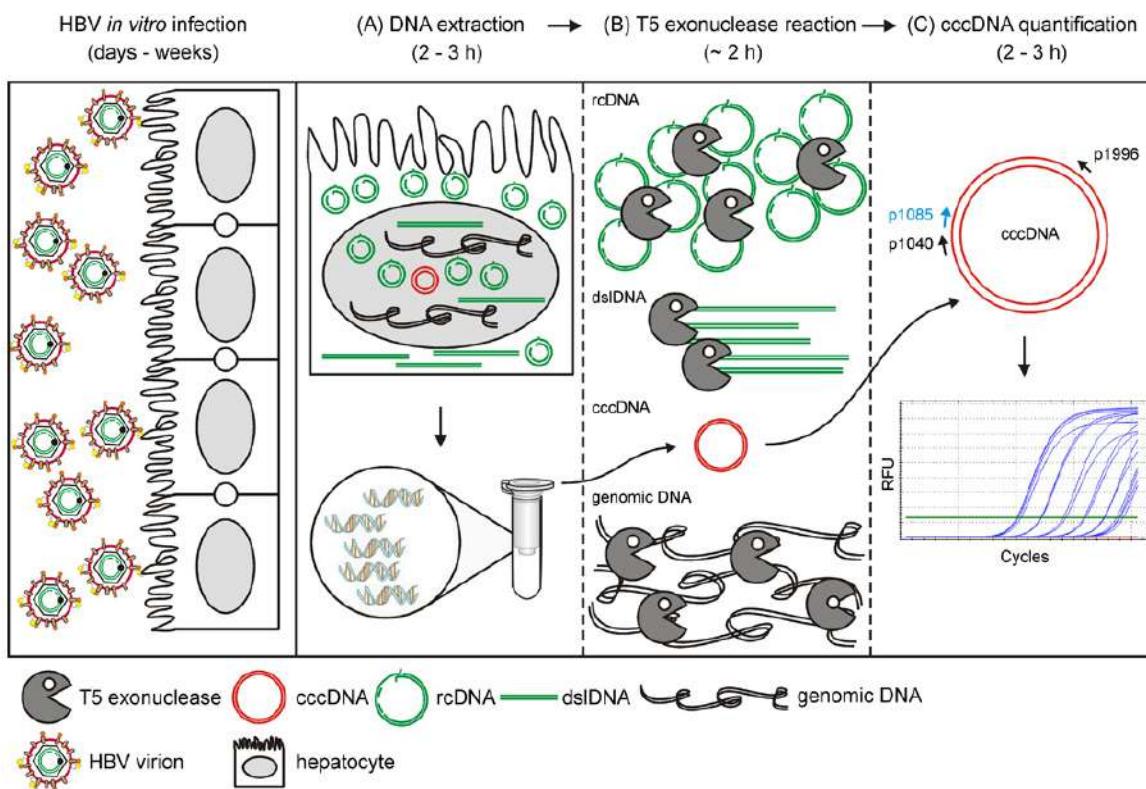


Figure 1. Working flowchart of this protocol. Susceptible cells (PHH, HepaRG^{NTCP}, HepG2^{NTCP}) infected with high mges (> 300) of HBV are lysed. A. Lysates are incubated at 70 °C in the presence of proteinase K and later loaded on silica column and eluted. B. T5 exonuclease (5 units/1 h) removes host genomic DNA (black) and HBV replicative intermediates (rcDNA, dsIDNA, etc.) (green) and preserves cccDNA (red) intact. C. cccDNA is amplified by p1040/p1996 and detected by probe p1085.

A. HBV DNA extraction from *in vitro* infected hepatocytes (Figure 1A)

- For infection performed in a 24-well plate (culture area: 2 cm²), trypsinize and resuspend infected PHH, differentiated HepaRG^{NTCP} or HepG2^{NTCP} cells (infection procedure see Ni and Urban, 2017) in 1 ml of culture medium. Take 10 µl of the resuspension for cell count by a cell counter (see Equipment). Calculate total cell numbers.

Notes:

- At confluence, cells in one well of a 24-well plate approximately correspond to 180,000 PHH, 200,000 HepaRG^{NTCP} and 500,000 HepG2^{NTCP} cells.
- When smaller or larger plates are used, volumes of the respective buffers given in the protocol should be adjusted according to the respective culture area (cm²) of a well.

- Spin down the cells (900 x g, 5 min) at room temperature. Wash cells with 1 ml of PBS twice. Spin down the cells and carefully aspirate PBS (Optional: freeze cell pellet at -20 °C for long-term storage). For DNA extraction, manufacturer's manual of the NucleoSpin Tissue kit

(740952.250) is adapted with a minor modification regarding the incubation time in Step A2 (1 h instead of 10~15 min) and the elution volume in Step A6 (50 μ l instead of 100 μ l). Add 200 μ l of lysis buffer T1, pipet up and down and incubate at room temperature for 10 min. Add 25 μ l of proteinase K and 200 μ l of lysis buffer B3, vortex and incubate the lysate at 70 °C for 1 h with shaking (500 rpm).

Note: After proteinase K digestion, it is not necessary to re-centrifuge the lysates. Immediately perform the next step.

3. Add 210 μ l of ethanol (96%-100%) to the sample and vortex vigorously for a few seconds. Remove buffer from the lid by a short centrifugation and apply the samples to the column. Centrifuge (11,000 $\times g$, 1 min) at room temperature. Discard flow-through.
4. Add 500 μ l of wash buffer BW. Centrifuge (11,000 $\times g$, 1 min) at room temperature. Discard flow-through.
5. Add 600 μ l of wash buffer B5 (pre-add indicated volume of ethanol to the Buffer B5 Concentrate) to the column. Centrifuge (11,000 $\times g$, 1 min) at room temperature. Discard flow-through. Repeat this step once.
6. Remove residual ethanol from the column (13,000 $\times g$, 1 min). Place the column into a 1.5 ml microcentrifuge tube. Add 50 μ l of pre-warmed (70 °C) elution buffer. Incubate at room temperature for 1 min. Centrifuge (11,000 $\times g$, 1 min). Freeze eluted DNA samples at -20 °C until use or analyze them immediately.

Notes:

- a. *Elution buffers are nuclease-free water or 5 mM Tris-HCl (pH = 8.0). Do not use buffers with EDTA that may inhibit enzymatic activity in the next step.*
- b. *DNA concentration in eluates is 200-1,000 ng/ μ l.*
- c. *Preserve at least 10 μ l of the sample without T5 exonuclease digestion for quantification of β -globin (internal standard) for normalization use.*

B. T5 exonuclease hydrolysis of total DNA elutes (Figure 1B)

1. Assemble the reaction components in a 0.2 ml microcentrifuge tube following the table as shown:

Component	Volume
10x NEBuffer 4	1 μ l
T5 exonuclease (10 U/ μ l)	0.5 μ l (5 U)
Crude DNA eluate (< 5 μ g)	5 μ l
Nuclease-free water	3.5 μ l
Total volume	10 μ l

Note: Do not exceed unit and incubation time of T5 exonuclease since overdigestion leads to partial loss of cccDNA.

2. Incubate the reaction at 37 °C for 1 h and inactivate the enzyme at 70 °C for 20 min in a thermocycler with heat-lid supply.

3. Proceed quantification by real-time qPCR or freeze the products at -20 °C until use.
- C. Quantification of cccDNA relative to human β-globin (single-copy gene) by real-time qPCR (Figure 1C)
 1. Quantification of cccDNA
 - a. Dilute primers and probe to 10 μM:

Primer/Probe	Sequence
Forward primer p1040	5'-GTGGTTATCCTGCGTTGAT-3' (see Notes section)
Reverse primer p1996	5'-GAGCTGAGGCCGGTATCT-3' (see Notes section)
Probe p1085	5'-FAM-AGTTGGCGAGAAAGTGAAAGCCTGC-TAMRA-3'

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine
 - b. Prepare the following qPCR reactions in a 96-well Hard-Shell PCR plate:

Component	Volume
2x PerfeCTa qPCR ToughMix	7.5 μl
p1040 (10 μM)	0.6 μl (final concentration: 400 nM)
p1996 (10 μM)	0.6 μl (final concentration: 400 nM)
p1085 (10 μM)	0.6 μl (final concentration: 400 nM)
DNA sample after T5 exonuclease treatment	2 μl #
Nuclease-free water	3.7 μl
Total volume	15 μl

#for absolute quantification, load 2 μl of each 1:10 serial-diluted plasmid (pSHH2.1: 10⁹, 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10² copies/μl) as templates on the same plate.
 - c. Run qPCR program below:

Step	Temperature	Time	Signal collection
1	95 °C	15 min	Off
2	95 °C	5 s	Off
3	63 °C	70 s	On

Repeat steps 2 and 3 for 50 cycles
 - d. Read absolute cccDNA copy numbers in the CFX Manager™ Software. Calculate cccDNA copies per well.
 2. Quantification of human β-globin prior to T5 exonuclease digestion
 - a. Dilute primers at 10 μM:

Primer	Sequence
Forward primer β -globin	5'-CAGGTACGGCTGTCATCACTTAGA-3'
Reverse primer β -globin	5'-CATGGTGTCTGTTGAGGTTGCTA-3'

- b. Prepare qPCR reactions in a 96-well plate:

Component	Volume
2x SYBR green Supermix	7.5 μ l
Forward primer (10 μ M)	0.6 μ l (final concentration: 400 nM)
Reverse primer (10 μ M)	0.6 μ l (final concentration: 400 nM)
DNA sample prior to T5 exonuclease treatment	2 μ l
Nuclease-free water	4.3 μ l
Total volume	15 μ l

- c. Start qPCR program below:

Step	Temperature	Time	Signal collection
1	95 °C	10 min	Off
2	95 °C	5 s	Off
3	60 °C	30 s	On
Repeat steps 2 and 3 for 40 cycles			

- d. Read levels of human β -globin. Normalize cccDNA copies per well using values of human β -globin. Calculate cccDNA copies per cell (copies per well divided by cell numbers per well). For instance, if cell numbers in a well of 24-well plate are 4.2×10^5 (as determined in Procedure A1) and cccDNA copies per well are 8.52×10^5 (as determined in Procedure C), cccDNA copies per cell are 2.03.

Data analysis

cccDNA copy number per cell or infected cell is calculated according to Figure 2. The cccDNA level in *in vitro* infected cells can be determined similarly as two examples shown in Figure 3 and Qu *et al.*, 2018. Note that to discriminate signals from the inoculum, we highly recommend using an entry inhibitor (e.g., Myrcludex B) as a control. Routinely Myrcludex B control gives a value below 0.05 copy per infected cell.

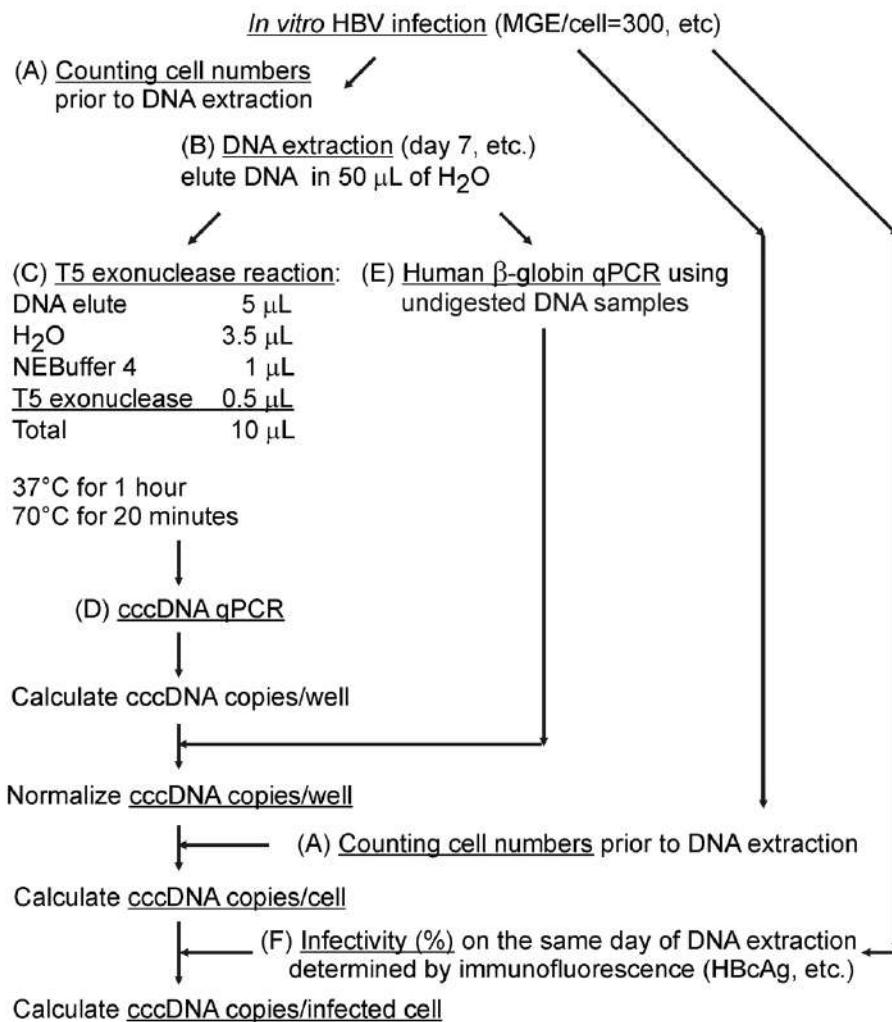


Figure 2. Schematic diagram of the calculation of absolute cccDNA copy numbers. A. On the day of experiment, cell number in the well is determined. B. Total DNA samples are extracted as suggested in Procedure. C. Here shows a proper T5 exonuclease digestion as shown in Procedure. D. Absolute cccDNA copies and E level of human β-globin are quantified, respectively as shown in Procedure. “cccDNA copies/cell” is “cccDNA copies/well” divided by “cell numbers”. Optional: F. If calculation of copies per infected cell is required, additional wells have to be arranged in parallel on the same plate during the infection. On the day of DNA extraction, cells in the wells are fixed and subjected to an immunofluorescence assay (HBcAg visualization using DAKO B0586 antibody, etc.) to determine infectivity (%: number of HBcAg positive cells divided by number of total cells) (Qu et al., 2018). “cccDNA copies/infected cell” is the “cccDNA copies/cell” divided by “infectivity (%)".

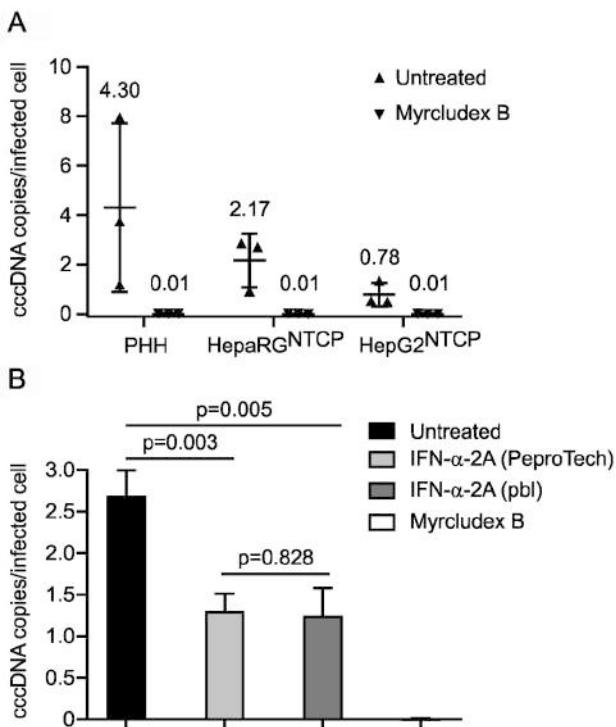


Figure 3. cccDNA levels in three *in vitro* models and upon IFN- α -2a and Myrcludex B treatment. A. PHH, differentiated HepaRG^{NTCP} and HepG2^{NTCP} cells were infected with HBV at mges/cell of 500. Myrcludex B (1 μ M), an entry inhibitor blocking cccDNA formation, was co-administered with HBV inoculum during infection. On Day 7 post infection, cccDNA copies per infected cell were determined. Data shown in triangles were collected from three independent experiments. B. HepG2^{NTCP} cells were infected with HBV at mges/cell of 500 with mock treatment (untreated), co-treated with Myrcludex B (1 μ M) during infection, or co- and post-treated with IFN- α -2A at 100 ng/ml purchased from PeproTech and PBL Assay Science. On Day 7 post infection, cccDNA copies per infected cell were analyzed. Statistics: $P < 0.01$ untreated versus IFN-treated; $P = 0.828$ PeproTech versus PBL.

Notes

The primer pair (pp1040-1996) is specific for the formed cccDNA in genotype D. Genotype D is the HBV genome in HepAD38 cell line. Users should consider this issue if they try patient-derived serum to perform *in vitro* infection using this protocol since HBV in patients can be any genotype and pp1040-1996 does not bind the formed cccDNA of other genotypes.

Genbank accession numbers of other genotypes: A (HE974370.1); B (AB540582.1); C (AB540584.1); E (HE974384.1); F (HE974369.1); G (AP007264.1); H (AB846650.1). pp1578-1867 and pp1583-2301 (Qu *et al.*, 2018) allowing quantification of eight HBV genotypes (A-H) are suggested below. (Table 1)

Table 1. List of primer mismatches to HBV genotypes. The numbers of base-pair mismatch of each forward and reverse primers to eight HBV genotypes are summarized below. Green squares: perfect binding; yellow squares: one base-pair mismatch still allowing template binding; red squares: not binding.

Mismatch Primer	HBV genotype							
	A	B	C	D	E	F	G	H
p1040 (F)	3	4	3	0	3	4	3	3
p1996 (R)(#)	2	3	2	0	0	3	4	5
p972 (F)	0	1	1	0	0	3	2	4
p1995 (R)	2	3	2	0	0	3	3	5
p1558 (F)	0	0	0	0	0	1	0	0
p1958 (R)	0	1	1	0	0	2	2	1
p1548 (F)	0	0	0	0	0	1	0	1
p1886 (R)	0	0	1	0	0	0	1	0
p1578 (F)	0	0	0	0	0	0	0	0
p1867 (R)	0	0	0	0	0	0	0	0
p1583 (F)	0	0	0	0	0	0	0	0
p2301 (R)	0	0	0	0	0	0	0	0

F: forward primer; R: reverse primer. #: The probe of this protocol is not pan-genotypic and has mismatch number as shown: A(1), B(2), C(0), D(0), E(0), F(6), G(1), H(4).

Recipes

1. Culture medium

For HepG2^{NTCP} cells: DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% MEM non-essential amino acids. For infection, add 2% dimethyl sulfoxide

For PHH and HepaRG^{NTCP} cells: William's medium E supplemented with 10% fetal calf serum (heat-inactivated), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% MEM non-essential amino acids, 5 µg/ml recombinant insulin, 50 µM hydrocortisone. For differentiation and infection, add 1.5% dimethyl sulfoxide

2. 10x PBS

Dissolve 80 g of NaCl, 2 g of KCl, 11.5 g of Na₂HPO₄·2H₂O and 2 g of KH₂PO₄ in 800 ml of H₂O, adjust pH to 7.4, refill H₂O to 1 L

3. Tris-HCl (1 M)

Dissolve 121.14 g of Tris-base in 800 ml of H₂O, adjust pH to 8.0 and refill H₂O to 1 L

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Competing interests

Prof. Dr. Stephan Urban, the corresponding author, holds patents and intellectual property on Myrcludex B.

Ethics

Following written informed consent of the patients, PHH were isolated from liver specimens obtained after partial hepatectomy.

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Analysis of Indole-3-acetic Acid (IAA) Production in Klebsiella by LC-MS/MS and the Salkowski Method

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[Abstract] Many rhizobacteria isolated from plant rhizosphere produce various phytohormones in the form of secondary metabolites, the most common of which is Indole-3-acetic acid (IAA). Here, we detail analytical protocols of IAA detection and quantification, *in vitro* and *in situ*, as recently applied to *Klebsiella* SGM 81, a rhizobacterium isolated from the rhizosphere of *Dianthus caryophyllus* (a commercially important flower across the globe). Specifically, we describe a detailed protocol for a colorimetric assay using the Salkowski reagent method, which can be used to screen for the presence of Indole compounds. To further detect and quantify IAA, a highly accurate analytical approach of LC-MS/MS is used. To detect the presence of IAA around the root system of *Dianthus caryophyllus*, *in situ* staining of plant roots is done using Salkowski reagent.

Keywords: Indole-3-acetic acid, *Klebsiella*, Salkowski reagent, LC-MS/MS, Tryptophan, Spectrophotometer

[Background] The bacterial auxin in the form of Indole-3 Acetic Acid (IAA) is a product of L-tryptophan metabolized by bacteria (Lynch, 1985). The group of bacteria known as plant growth promoting rhizobacteria (PGPR) specifically residing in the vicinity of the roots depend on tryptophan being present in the root exudates of plants (Kravchenko *et al.*, 2004; Kamilova *et al.*, 2006). These PGPR use IAA as a signal to interact with plant roots and to colonize the plant parts. This signaling feature of IAA is thought to effect on the physiology of the bacteria (Spaepen *et al.*, 2007).

Different methods are found in the literature to detect the biosynthesis of IAA. Gordon and Weber, (1951) were the first to provide a colorimetric assay using Salkowski reagent for the detection of IAA. This method has since been widely used for detecting IAA from microorganisms. Salkowski reagent is a mixture of 0.5 M ferric chloride (FeCl_3) and 35% perchloric acid (HClO_4) which upon reaction with IAA yields pink color, due to IAA complex formation with and reduction of Fe^{3+} (Kamnev *et al.*, 2001). The color developed by positive reaction indicates the presence of various indole compounds as a product of tryptophan metabolism. Apart from the colorimetric assay, other methods for IAA estimation from bacteria and plant are High Performance Liquid Chromatography (HPLC) (Perrig *et al.*, 2007), Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS/MS) (Chiwocha *et al.*, 2003), and by High Performance Thin Layer Chromatography HPTLC (Goswami *et al.*, 2015). Liquid Chromatography (LC) is the preferred approach to determine the concentration of IAA and to confirm its purity with high accuracy and standardization. LC coupled with various mass spectrometry

detectors are powerful tools for IAA analysis. Because of the high sensitivity and selectivity, Mass Spectrometry detectors are most commonly coupled with LC. One of the important benefits of LC-MS is that analysis and separation of compounds can be achieved in a continuous manner eliminating the step of purification (Kallenbach *et al.*, 2009).

Materials and Reagents

1. 100-1,000 µl pipette tips (Sigma-Aldrich)
2. Test tube racks (Sigma-Aldrich, catalog number: Z334146)
3. Screw-cap tubes and caps (Sigma-Aldrich, catalog number: AXYSCT10MLS)
4. Pyrex test tube 20 ml (Sigma-Aldrich, catalog number: CLS980016)
5. UV quartz cuvette semi-micro square, 1.4 ml, PTFE stopper (Sigma-Aldrich, catalog number: Z276707)
6. Sterile syringe filters with a pore size of 0.2 µm (Fisher Scientific, catalog number: 720-1230)
7. Centrifuge tube with screw cap, capacity 10 ml (Sigma-Aldrich, catalog number: SIAL301NN10R)
8. Pyrex glass serological pipettes, capacity 5 ml (Sigma-Aldrich, catalog number: Z653829)
9. Amber storage bottles (Sigma-Aldrich, Supelco, catalog number: 23230-U)
10. *Klebsiella* SGM 81 strain (isolated from the rhizosphere of *Dianthus caryophyllus*)
11. Distilled water
12. Nutrient broth (Microbiology grade) (Sigma-Aldrich, catalog number: 70122-500G), its composition and final pH see below:

Grade	For microbiology
Composition	D(+)-glucose, 1 g/L
	Peptone, 15 g/L
	Sodium chloride, 6 g/L
	Yeast extract, 3 g/L
Final pH	7.5 ± 0.2 (25 °C)

13. FeCl₃ reagent grade (Sigma-Aldrich, CAS: 7705-08-0)
14. Perchloric acid 70% (Sigma-Aldrich, CAS: 7601-90-3)
15. Methanol (laboratory grade) (Sigma-Aldrich, CAS: 67-56-1)
16. HCl (ACS grade) (Sigma-Aldrich, CAS: 7647-01-0)
17. L-tryptophan (traceCERT grade) (Sigma-Aldrich, CAS: 73-22-3)
18. Acetic acid glacial (Sigma-Aldrich, CAS: 64-19-7)
19. Ethyl acetate (grade anhydrous) (Sigma-Aldrich, CAS: 141-78-6)
20. Indole-3-Acetic acid (IAA) (Sigma-Aldrich, CAS: 87-51-4)

21. Murashige and Skoog medium (Sigma-Aldrich)
22. Acetonitrile
23. Ammonium formate
24. CH₃CN
25. Formic acid
26. Ethanol
27. Agarose
28. Culture media (see Recipes)
29. Salkowski reagent (see Recipes)

Equipment

1. Spatula (Thermo Fisher, catalog number: F36711-0012)
2. Nichipet Eco pipette, 100-1,000 µl volume (Sigma-Aldrich, catalog number: Z710199)
3. Measuring cylinder (Sigma-Aldrich, catalog number: Z324361-2EA)
4. 250 ml conical flask (Sigma-Aldrich, catalog number: Z723088-1EA)
5. Rotary Vacuum Flash Evaporator (Buchhi Type) (Jain Scientific Glass, 1188)
6. Balance
7. Agilent 1100 LC system and an ABSciex 6500 Qtrap MS [Chromatography was on a Phenomenex Luna C18 column (100 mm x 2 mm x 3 µm)]
8. UV-spectrophotometer (Systronics, 166)
9. Microcentrifuge (Thermo scientific, Pico 17)
10. Magnetic stirrer (Remi, 1 MLH)
11. Vortex cyclo mixer (Remi, CM 101)

Software

1. Analyst 1.6.1 (AB Sciex)

Procedure

- A. IAA screening and quantification from *Klebsiella* SGM 81 using Salkowski reagent method
 1. Culture preparation
 - a. Take preculture medium (without tryptophan) and inoculate it with a single colony of SGM 81 strain, or a loopful of glycerol stock of the strain.
 - b. Incubate the inoculated pre-culture medium at 30 °C, 120 rpm overnight. This will result in the young culture of the *Klebsiella* SGM 81 strain which can be used for inoculating IAA production medium (test media).
 - c. Add 100 µl of the young culture in test medium and vortex it to get a uniform suspension.

- d. Incubate it under dark conditions (by wrapping the container with newspaper/aluminum foil) at 30 °C in shaking condition at 120 rpm.
 - e. Incubation lasts for 24-96 h in dark condition depending on IAA production ability of the strain. *Klebsiella* SGM 81 produces IAA till 72 h and subsequent decrease in the production is recorded after that.
2. Indole quantification using Salkowski reagent
 - a. After 24 h, take out 1.5 ml of sample culture broth and transfer to an Eppendorf tube.
 - b. Centrifuge at 16,278 $\times g$ for 5 min using a microcentrifuge.
 - c. Carefully withdraw 1 ml supernatant and transfer to a new test tube.
 - d. Mix equal volume (1 ml) of Salkowski reagent, vortex gently and incubate the reaction at 30 °C in a dark condition for 30 min.
 - e. In a new test tube replace the supernatant with 1 ml of control uninoculated medium and mix with 1 ml of Salkowski reagent. This serves as blank.
 - f. The presence of IAA is detected by measuring pink color development after 30 min (some strains may develop red color as shown in Figure 1).

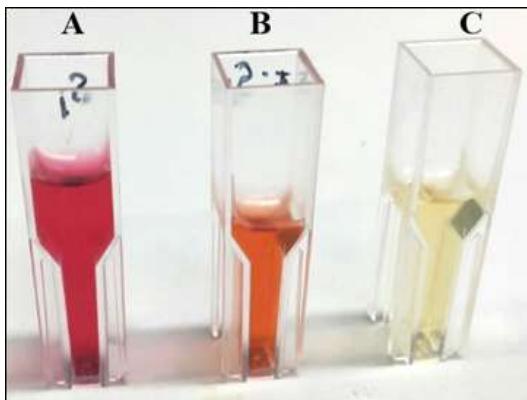


Figure 1. Color development due to Indole-Salkowski reagent reaction. A. *Klebsiella* SGM 81. B. SGM09. C. Control uninoculated media.

- g. Use uninoculated medium to set the blank. Measure the color intensity spectrophotometrically at 536 nm using cuvette.
 - h. Compare the optical density of the test sample with a standard IAA curve ($10\text{-}100 \mu\text{g}\cdot\text{ml}^{-1}$) to calculate the concentration.
 - i. Repeat the process at similar intervals until the optical density begins to decrease, indicating no further production of IAA.
- B. Analysis of IAA by liquid chromatography-mass spectrometry (LC-MS/MS)
 1. Sample preparation
 - a. Prepare 100 ml nutrient broth medium supplemented with 0.5 g of L-tryptophan in a 250 ml Erlenmeyer flask.

- b. Add 100 μ l of the young culture in test medium and vortex it to get a uniform suspension.
 - c. Incubate it under dark conditions (by wrapping the container with newspaper/aluminum foil) at 30 °C in shaking condition at 120 rpm for 72 h.
 - d. To separate the cells, centrifuge at 4 °C for 20 min at 2,800 $\times g$ and collect 1,000 ml supernatant.
 - e. Transfer the supernatant to a 250 ml screw-cap glass bottle. Acidify it by adding 2-3 drops of 1 N concentrated HCl to reach pH 2.5-3.
 - f. To extract IAA, add double the volume of ethyl acetate to the acidified supernatant and shake vigorously for 5 min (alternatively, a separating funnel can be used). Let the mixture stand at room temperature for 10 min to get the top layer of ethyl acetate. Use this layer for further treatment.
 - g. In a rotary evaporator, set water bath temperature to boiling temperature of ethyl acetate i.e., 77.1 °C (alternatively, ethyl acetate phase can be vacuum dried in a rotational evaporator at 40 °C). The time for drying of the solvent system varies according to the sample volume.
 - h. Transfer the ethyl acetate layer to the round bottom flask and adjust its rotation to avoid any bumping of the liquid sample. “Bumping is the phenomenon in chemistry where homogenous liquids boiled in a test tube or other container will super heat and, upon nucleation rapid boiling will expel the liquid from the container (Wikipedia)”–This would leave the IAA escaping the flask into the condenser tube attached with the round bottom flask.
 - i. Upon complete evaporation of the liquid ethyl acetate, pure IAA is left behind in the crystalline form attached to the bottom of the rotary flask.
 - j. Switch off all the units of the rotary evaporator and remove the round bottom flask. Re-dissolve the crystalline IAA in 5 ml of 20% methanol and store at -20 °C for future use.
2. LC-MS/MS analysis
 - a. Filter the stored methanol extract (100 μ l) using sterile syringe filters of 0.2 μ m so as to separate insoluble particles and larger compounds if any. This is the final analysis sample.
 - b. Phenomenex Luna C18(2) column (100 mm x 2 mm x 3 μ m) at temperature of 50 °C is used as chromatography system.
 - c. A gradient solvent system of 10% solvent A (2% acetonitrile, 10 mM ammonium formate, pH 4.2) to 90% solvent B (94.9% CH₃CN, 5% H₂O, 0.1% formic acid) is used for the column over 10 min at a flow rate of 250 ml/min.
 - d. Wash the column with 90% solvent B for 3 min and then re-equilibrate with 90% solvent A for 6 min.
 - e. Use 10 μ l injections to load the sample for analysis.
 - f. The MS is configured with a Turbo Spray Ion Drive source where the source temperature is set to 500 °C and the ion spray voltage to 5,500 V.
 - g. Analyze IAA by Multiple Reaction Monitoring (MRM) in positive mode using a transition of 176 > 130 with collision energy of 20 eV. Transition of 176 > 130 means the specific pairs

of mass to charge (m/z) values associated to the precursor and fragment ions. In our case the Q1 is set to a value of 176 amu and the Q3 to 130 amu, the collision energy was 20 mV.

- h. Set the declustering potential, exit potential and collision cell exit potential at 30 V, 10 V and 10 V respectively.

C. *In Situ* Salkowski staining

1. Prepare Murashige and Skoog (MSM) basal salt medium with 0.8% agarose, devoid of plant hormone supplements.
2. Sterilize the *D. caryophyllus* seeds using three-step procedure: a 1 min wash in 70% ethanol, followed by a 4 min wash in 20% NaClO, and a final rinse in sterile distilled water 3 times.
3. Allow the *D. caryophyllus* seeds to germinate on the MS media by incubating the plates at 25 °C in 14 h light and 10 h dark cycles in a plant culture room, until the root length reaches 2 cm (approximately six days).
4. Treat the germinated plant roots by immersing the root tips into 5 ml of the bacterial suspension with titre 10^5 CFU ml $^{-1}$ in the universal tube.
5. Transfer the treated plants onto new MS agar medium devoid of IAA and sucrose in square Petri plates (120 mm x 120 mm x 15 mm).
6. Again, incubate the plates at 25 °C in 14 h light and 10 h dark cycles in a plant culture room.
7. After two weeks of infection, stain the treated roots with Salkowski reagent.
8. To stain the roots, add 400 μ l of Salkowski reagent on each root and observe any visible color change.
9. The development of pink color as shown in the figure below indicates the presence of proximal IAA on and around the roots (Figure 2).



Figure 2. *In situ* Salkowski staining on plant roots treated with *Klebsiella* SGM 81 of 10^5 CFU ml $^{-1}$ titre

Data analysis

1. Take the optical density of the samples at 536 nm and graphically calculate the concentration of IAA based on the standard curve. IAA estimation from *Klebsiella* SGM 81 strain, using

Salkowski reagent shows a maximum yield to $215 \mu\text{g}\cdot\text{ml}^{-1}$ detected after 72 h with 0.15% tryptophan (Figure 1a in Gang et al., 2018).

2. For LC-MS/MS analysis, confirmation of the identity in the samples is done by Enhanced Product Ion scans.
3. Data acquisition and analysis is done with Analyst 1.6.1 (AB Sciex).
4. Quantification is carried out by comparing the test sample data with control standard IAA.
5. Overlapping retention times during LC (5.90 min and 5.92 min) and identical m/z ratios (130.1) between a commercial IAA standard and sample supernatant showed *Klebsiella* SGM 81 to produce substantial amounts ($960 \mu\text{g}\cdot\text{ml}^{-1}$ after 72 h) homogeneous IAA (Gang et al., 2018).
6. Development of pink color on the Salkowski treated roots indicates the presence of bacterial IAA proximal to the roots (Figure 3a in Gang et al., 2018).

Notes

1. The Salkowski reagent method discussed in this paper is shown using nutrient broth liquid medium (supplemented with L-tryptophan) which is non-specific liquid medium for most gram-positive and gram-negative bacteria. However other media like Yeast extract mannitol (YEM), Pikovskaya's broth (supplemented with L-tryptophan) can be used if IAA production and quantification is to be done from Rhizobium or phosphate solubilizing bacteria respectively.
2. The preculture preparation is important to normalize the initial cell count especially when a comparative study of IAA quantification is carried out for the optimization process.
3. Salkowski reagent method quantifies the overall Indole present in the sample. To get the purity and conjugants of Indole-3-acetic acid (IAA), other analytical methods like HPLC, LC-MS/MS, HPTLC etc. can be used.

Recipes

1. Culture media
 - a. Making use of measuring cylinder, measure 100 ml distilled water and transfer it into a 250 ml Erlenmeyer flask or screw-capped glass bottle. Two other flasks of the same media, with and without tryptophan will be used as control and pre-culture media (used to activate the SGM 81 culture to yield young culture) respectively
 - b. For simple liquid nutrient broth medium, weigh 1.3 g of Nutrient broth and dissolve it in 100 ml distilled water. Stir it properly so as to mix the powder in distilled water properly
 - c. Weigh 0.15 g of L-tryptophan (0.15% w/v) and add to the prepared nutrient broth flask. This is liquid nutrient broth with tryptophan supplement
 - d. Autoclave the prepared media at 121 °C for 15 min
 - e. Allow the media to cool down and come to room temperature before inoculation so as to ensure the survival of inoculum

2. Salkowski reagent
 - a. 0.5 M of 100 ml ferric chloride (dissolve 8.125 g of FeCl₃ in 100 ml of distilled water)
 - b. To dilute readily available perchloric acid, measure 24.5 ml (v/v) of distilled water in a measuring cylinder and add 24.5 ml (v/v) of concentrated acid to it
 - c. Add 1 ml (v/v) of 0.5 M ferric chloride solution to 49 ml of 35% perchloric
 - d. Mix well and store in a dark brown bottle at room temperature. It can be used for further experiments if stored under described conditions

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Competing interests

We declare no conflict of interest.

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Enterovirus Competition Assay to Assess Replication Fitness

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[Abstract] In virology the difference between the fitness of two viruses can be determined by using various methods, such as virus titer, growth curve analysis, measurement of virus infectivity, analysis of produced RNA copies and viral protein production. However, for closely performing viruses, it is often very hard to distinguish the differences. *In vitro* competition assays are a sensitive tool for determining viral replication fitness for many viruses replicating in cell culture. Relative viral replication fitness is usually measured from multiple cycle growth competition assays. Competition assays provide a sensitive measurement of viral fitness since the viruses are competing for cellular targets under identical growth conditions. This protocol describes a competition assay for enteroviruses and contains two alternative formats for initial infections, which can be varied depending on specific goals for each particular experiment. The protocol involves infection of cells with competing viruses, passaging, RNA extraction from infected cells, RT-PCR and Sanger sequencing followed by comparative analysis of resulting chromatograms obtained under various initial infection conditions. The techniques are applicable to members of many virus families, such as alphaviruses, flaviviruses, pestiviruses, and other RNA viruses with an established reverse genetics system.

Keywords: Enterovirus, Competition assay, Virus titration, Dual infection, Pairwise growth competition assay, Virus passaging, Sanger sequencing

[Background] Enteroviruses comprise a large group of mammalian pathogens that includes poliovirus. Pathology in humans ranges from sub-clinical to acute flaccid paralysis, myocarditis and meningitis. In our recent paper (Lulla *et al.*, 2019) we reported that many enterovirus genomes harbor an upstream open reading frame (uORF) that encodes an additional viral protein, UP (upstream protein). Using the echovirus 7 (EV7) reverse genetics system, we created two UP knock-out mutants (EV7-Loop and EV7-PTC), which exhibited wtEV7-like growth properties. Therefore, we decided to perform a competition assay to determine possible subtle fitness defects.

Materials and Reagents

A. Materials

1. Pipette tips (Fisher, catalog numbers: 02-707-426, 02-707-403, 02-707-438)
2. 1.5 ml microfuge tubes (STARLAB, catalog number: 51615-5500)
3. T175 tissue culture flasks (TPP® tissue culture flasks, Sigma, catalog number: Z707562)
4. 12-well polystyrene culture plates (TPP® tissue culture plates, Sigma, catalog number: Z707775)

B. Viruses

Echovirus 7 (EV7), derived from EV7 infectious clone

Note: The cDNA of Echovirus 7 strain Wallace was sourced from Michael Lindberg (GenBank accession number AF465516, with the silent substitution ¹⁶⁸⁷G-to-A) and was cloned downstream of a T7 RNA promoter. Mutant viruses EV7-Loop and EV7-PTC were generated by mutagenesis of the original EV7 infectious clone and prepared exactly as wt EV7 (Lulla et al., 2019).

C. Cell lines

RD cells (human rhabdomyosarcoma cell line, ATCC, CCL-136), verified mycoplasma-free by next-generation sequencing

D. Reagents

1. Dulbecco's modified Eagle's medium (DMEM) (Sigma, catalog number: D6546)
2. Fetal bovine serum (FBS) (PAN Biotech, catalog number: P40-37500)
3. Penicillin-streptomycin (10,000 U/ml) (Life Technologies, catalog number: 15140-122)
4. 200 mM L-Glutamine (Gibco, catalog number: 25030081)
5. Phosphate buffered saline (PBS) (Life Technologies, catalog number: 14190-144)
6. 1 M HEPES buffer, pH range: 7.2-7.5 (Life Technologies, catalog number: 15630-080)
7. 0.25% Trypsin (Gibco, catalog number: 15090046-100)
8. Bovine serum albumin (BSA) (PAN Biotech, catalog number: P06-1395500)
9. Direct-zolTM RNA MiniPrep Plus (Zymo Research, catalog number: R2072)
10. PhusionTM RT-PCR Kit (Thermo Scientific, catalog number: F-546S)
11. Complete DMEM (see Recipes)
12. Serum-free DMEM (see Recipes)

Equipment

1. -80 °C freezer
2. Vortexer
3. Pipettes: 1000 µl, 200 µl, 20 µl, 2 µl
4. CO₂ incubator
5. Haemocytometer
6. BSL2 cell culture cabinet
7. Rocking platform shaker
8. Phase-contrast inverted microscope (Nikon TMS)
9. 4 °C refrigerator
10. Autoclave

Software

1. BioEdit (Free software, Ibis Therapeutics, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>)

Procedure

IMPORTANT NOTE: All experiments with viruses (i.e., all pre-Trizol RNA isolation steps) should be performed inside a biosafety level 2 (BSL2) tissue culture laboratory according to the country and institution regulations and required permits regarding enterovirus strains handling and storage.

A. Infection and virus passaging

1. Prepare cells for infection 1 day before by seeding 3×10^5 RD cells per well on 12-well plates (the aim is to achieve 5×10^5 RD cells per well by the next day, which corresponds to subconfluent RD monolayers).
 - a. RD cells seeded on T175 flasks and grown in complete DMEM until 80-90% confluence. Aspirate media from the cells and rinse with 10 ml of PBS. Trypsinize RD cells using 3 ml of 0.25% trypsin by gently tapping the flask.
 - b. Once cells are detached, add 10 ml of complete DMEM, ensure the cells are properly resuspended by pipetting up and down. Count cells in suspension using hemocytometer.
 - c. Dilute cell suspension with complete DMEM to get concentration corresponding to 3×10^5 cells per 1 ml. Seed 1 ml of cell suspension to each well of 12-well plates.
 - d. Incubate overnight in 37 °C, 5% CO₂ incubator.
2. Check the cell monolayers using a microscope (equal healthy cell monolayers, no CPE). Remove media from cell monolayers and infect with a total multiplicity of infection (MOI) of 0.1, in 0.2 ml of serum-free DMEM. Use the following formula to calculate the amount of virus needed:

$$\text{Volume of virus stock (in } \mu\text{l)} = \frac{\text{Number of cells} \times \text{MOI} \times 10^3 \mu\text{l/ml}}{\text{Virus titer (PFU/ml)}}$$

Example: For 10⁹ PFU/ml virus stock use, 5×10^5 cells \times 0.1 PFU/cell \times 10³ $\mu\text{l/ml}$ / 10⁹ PFU/ml = 0.05 μl .

3. Mix using wt EV7 and mutant at either equal or 1:9 proportions, with a total MOI 0.1.

Note: This assay can be completed with other viruses and cell lines. Different proportions and MOI can be used for this experiment depending on the virus growth kinetics and susceptibility of cells for infection. Using MOI 0.1 means that more than 90% of the cells remain uninfected providing enough space for competing viruses; any growth difference should also accumulate over passaging.
4. Use triplicates for each infection, monoinfections for positive controls and mock (uninfected) cells as negative controls as suggested in Figure 1.

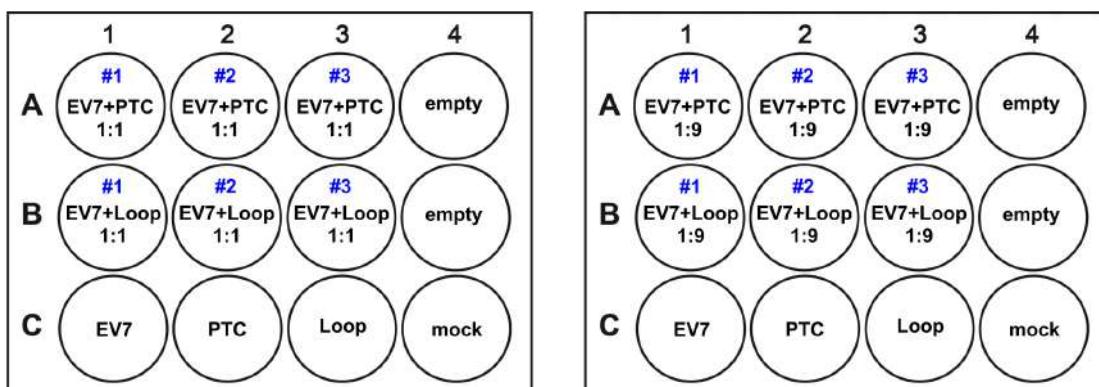


Figure 1. Sample layout of two 12-well plates at either equal (Left) or 1:9 (Right) proportions. Every subsequent passage is performed on similarly arranged 12-well plates.

5. Incubate infected cells at room temperature on a gently rocking platform for 1 h. To remove unbound virus, aspirate infection media, wash each well with 1 ml of serum-free DMEM, and overlay cells with 0.5 ml of serum-free DMEM.
6. Incubate at 37 °C in a CO₂ incubator overnight (16-20 h) until complete CPE is observed.
7. Collect media from infected plates to a 1.5 ml tube, centrifuge at 9,600 × g for 5 min, then transfer to the new 1.5 ml tube and measure the volume.
8. Add 0.2% BSA to supernatant (10 µl of 10% BSA per 0.5 ml of clarified supernatant).
9. Perform 5 blind passages (with estimated, but unknown titer) using 1:10,000 volume of obtained virus stock (corresponds to MOI 0.05-0.2). Use the same infection protocol as before (steps 1-8).

Note: For less efficiently replicating viruses more than 5 passages could be needed.

10. Store virus stocks at -80 °C.
11. Add 1 ml PBS to remaining cells and collect them by pipetting into a 1.5 ml tube (infected cells detach easily and do not require scraping). Centrifuge cells at 9,600 × g for 5 min, remove supernatant and proceed to RNA isolation from the infected cells.

B. RNA isolation from infected cells

1. Isolate RNA from passages 1 and 5 using Direct-zol™ RNA MiniPrep Plus (Zymo Research) according to the manufacturer's protocol ([Direct-zol™ RNA MiniPrep Plus INSTRUCTION MANUAL ver.1.10.1](#)).
2. Perform RT-PCR using Thermo Scientific™ Phusion™ RT-PCR Kit using virus-specific primers and manufacturer's recommendations ([Thermo Scientific Phusion RT-PCR Kit manual](#)). The example design of the introduced mutations and sequencing primer is presented in Figure 2.

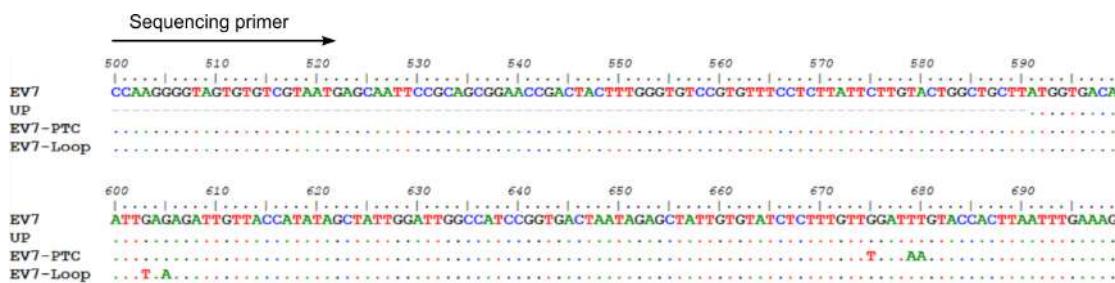


Figure 2. Example design of mutant virus genomes and sequencing primer. Depending on sequencing facilities, a 300-1000 bp long PCR fragment should be optimal for this assay.

3. Perform Sanger sequencing of the PCR fragment containing the mutated region of the virus genome.

Note: Please consider the nature of the virus (intracellular or budded virions) for choosing the correct sample to analyze. Sometimes analyzing RNA from media rather than from infected cells can be considered. For enteroviruses it is not a crucial step; therefore an easier approach (RNA isolation from infected cells) was chosen.

Data analysis

Compare chromatograms and evaluate based on three RT-PCR products from each analyzed sample (Figure 3). In the experimental conditions tested viruses do not differ in their growth properties. The chromatograms should shift if the viruses differ in their growth properties. Compare the peak areas from at least three different chromatograms for each sample and estimate the changes. If wt virus out-competes the mutant, the chromatogram of the fifth passage sample should match the wt virus chromatogram.

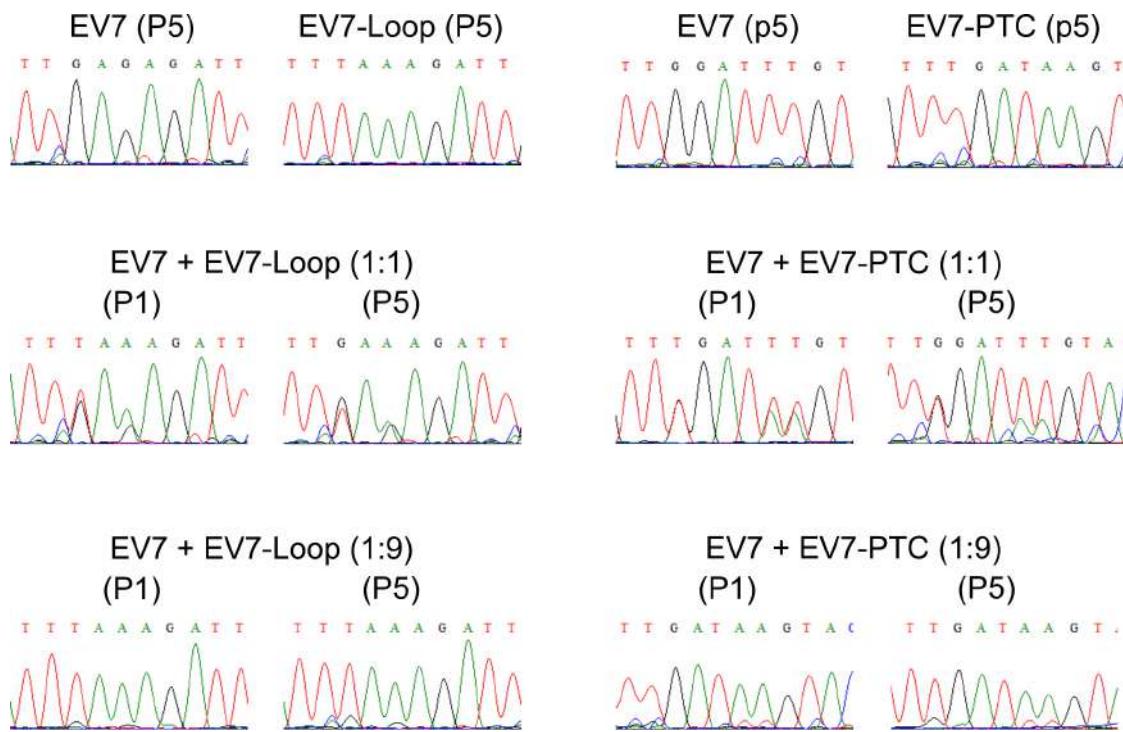


Figure 3. Sample sequencing chromatograms of RT-PCR products for the competition.
Experiment of EV7, EV7-PTC and EV7-Loop mutants at initial infection (P1) and after five passages (P5). The figure is reproduced from Supplementary Figure 3 in Lulla *et al.* (2019).

Recipes

1. Complete DMEM
500 ml DMEM
50 ml FBS
5 ml 200 mM L-Glutamine
5 ml penicillin-streptomycin (10,000 U/ml)
10 ml 1 M HEPES pH 7.2-7.5
Store at 4 °C
2. Serum-free DMEM
500 ml DMEM
5 ml 200 mM L-Glutamine
5 ml penicillin-streptomycin (10,000 U/ml)
10 ml 1 M HEPES, pH 7.2-7.5
Store at 4 °C

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Competing interests

The authors declare that they do not have any conflicts of interests or competing interests.

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TZA, a Sensitive Reporter Cell-based Assay to Accurately and Rapidly Quantify Inducible, Replication-competent Latent HIV-1 from Resting CD4⁺ T Cells

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[Abstract] The latent HIV-1 viral reservoir in resting CD4⁺ (rCD4⁺) T cells represents a major barrier to an HIV-1 cure. There is an ongoing effort to identify therapeutic approaches that will eliminate or reduce the size of this reservoir. However, clinical investigators lack an assay to determine whether or not a decrease in the latent reservoir has been achieved. Therefore, it is critical to develop assays that can reproducibly quantify the reservoir size and changes therein, in participant's blood during a therapeutic trial. Quantification of the latent HIV viral reservoir requires a highly sensitive, cost-effective assay capable of measuring the low frequency of rCD4⁺ T cells carrying functional provirus. Preferably, such an assay should be such that it can be adopted for high throughput and could be adopted under conditions for use in large-scale clinical trials. While PCR-based assays are commonly used to quantify pro-viral DNA or intracellular RNA transcript, they cannot distinguish between replication-competent and defective proviruses. We have recently published a study where a reporter cell-based assay (termed TZA or TZM-bl based quantitative assay) was used to quantify inducible replication-competent latent HIV-1 in blood. This assay is more sensitive, cost-efficient, and faster than available technology, including the quantitative viral outgrowth assay or the Q-VOA. Using this assay, we show that the size of the inducible latent HIV-1 reservoir in virally suppressed participants on ART is approximately 70-fold larger than previous estimates. We describe here in detail an optimized method to quantitate latently infected cells using the TZA.

Keywords: TZM-bl cells, TZA assay, Latent reservoir, Latent HIV-1, Quantification of latent reservoir, Inducible virus, Replication competent virus

[Background] The ability to quantitate the latent HIV-1 viral reservoir in a combination ART suppressed individual requires a highly sensitive assay with the ability to measure low frequency of rCD4⁺ cells carrying functional provirus. This assay should also be cost-effective and adaptable to be high throughput under different conditions and large-scale clinical applications. Currently, the frequency of the infected cells in latent condition is estimated using Poisson Statistics or by maximum likelihood analysis (Cillo *et al.*, 2014; Rosenbloom *et al.*, 2015). Most of these methods including the original Q-VOA method and its adaptations use limited dilution based techniques of PHA simulated CD4⁺ T cells which either measures HIV-1 protein or RNA via ELISA or quantitative PCR. These are all labor-intensive, time-consuming, expensive, and requires the frequent addition of activated CD4⁺ T cells as feeders (Chun *et al.*, 1997; Finzi *et al.*, 1999; Siliciano *et al.*, 2003; Siliciano and Siliciano, 2005). There has also been a modified version of Q-VOA assay reported which implements MOLT-4/CCR5 (Laird *et*

al., 2013) cell line for viral expansion instead of activated CD4⁺ T cells followed by RNA measurement but this is still more time consuming and labor intensive.

We recently reported the development of a sensitive TZM-bl cell-based assay (termed TZA) (Sanyal *et al.*, 2017) to quantify the latent HIV-1 reservoir in blood. The present protocol consists of these main parts (i) isolation of resting T cells from the blood from HIV-1 positive and negative (control) donors (Steps A1-A4); (ii) activation of these resting CD4⁺ T cells using a strong LRA like Anti-CD3/CD28 (Siliciano and Siliciano, 2004) antibodies and (iii) washing, counting and plating these activated cells on a TZM-bl reporter cells to quantitate replication-competent HIV-1 by measuring β-gal expression to quantify virus induced. This assay utilizes the TZM-bl cell line, which stably expresses CD4, CCR5, and CXCR4, and carries an integrated copy of the β-galactosidase (β-gal) gene under the control of an HIV-1 long terminal repeat (HIV-1 LTR) promoter that allows for the detection of inducible replication-competent HIV-1. By using TZM-bl cells, quantification of replication-competent HIV-1 can be achieved with high sensitivity (Ananworanich and Mellors, 2015). TZA has been used to calculate fraction of inducible latent virus (fPVE) (Cillo *et al.*, 2014; Sanyal *et al.*, 2017). The level of fPVE can be used to screen various LRAs *in-vitro* as well as help monitor their *in-vivo* efficacy especially during clinical trials. This assay reveals that the viral reservoir is likely much larger than previously predicted and estimated which can impact the ongoing therapeutic approaches to eradicate HIV-1.

This assay can be adapted and automated to a 384-well format further down the line to enable an efficient screening platform, which will be capable of handling multiple samples simultaneously. This will make it a high-throughput assay system. In addition, because of the low cell requirement in this system, the TZA may also be useful for quantification of replication-competent HIV-1 in the pediatric population as well as estimation of the reservoir in tissues. We are presently developing a protocol for determining the reservoir size in tissues using this assay.

Materials and Reagents

1. Pipette tips
2. 15 ml centrifuge tubes (Fisherbrand, catalog number: 07-200-886)
3. 50 ml centrifuge tubes (Fisherbrand, catalog number: 0553913)
4. Tissue culture plates, 24-well transparent (Falcon, catalog number: 353047)
5. White, 96-well plates, sterile with white lids (PerkinElmer, catalog number: 6005181)
6. EDTA tubes purple tops for blood collection: Vacutainer brand sterile (BD, catalog number: 366643)
7. CryoTube™ vials for freezing cells (Thermo Fischer Scientific, catalog numbers: 153779, 202209, 363401)
8. Tissue culture plates, 48-well transparent (Falcon, catalog number: 353078)
9. 5 ml round-bottom polystyrene tubes (Corning, Falcon®, catalog number: 352054)
10. TZM-bl cells (NIH AIDS Reagent program, catalog number: 8129)

11. Animal serum complex (Gemini Bio Products, Fetal Plex™, catalog number: 100-602), storage in a -18 °C freezer before opening bottle and then store at 4 °C for subsequent use
12. DMSO (Life Technologies Corp, catalog number: 20688)
13. FBS (HyClone, catalog number: SH3011803)
14. Beta-Glo Assay System (Promega, catalog number: E4780), storage in a -18 °C freezer
15. Custom ordered resting CD4⁺ T cells negative selection kit (Stemcell Technologies, catalog number: 19309VK) with EasySep D Magnetic Particles (Stemcell Technologies, catalog number: 19250), storage at 4 °C
16. Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fischer Scientific, Gibco™, catalog number: 11131D)
17. Clinical grade Human Recombinant Interleukin-2: IL-2 Powder 22 MU VIAL (65483011607), storage in a -18 °C freezer where MU stands for medical units
Note: Alternatively, human recombinant IL-2 can be purchased from Life Technologies Corp, catalog number: PHC-0023.
18. Human Recombinant Interleukin-7: IL-7 Premium grade (Miltenyi Biotec, catalog number: 130-095-361), storage in a -18 °C freezer
19. Efaviranz (EFV) (Cayman Chemical, catalog number: 14412), need to be re-suspended in IMDM media and diluted according to need of the experimental procedure
20. Flow Monoclonal Antibodies
Note: You will use 5 µl of each antibody for staining. Use simple Flow staining protocol for staining the cells. This is just done to check for the purity of the resting T cells. Resting T cells are CD25/CD69/HLADR⁻ cells.
 - a. Mouse Anti-human CD4-AF700 (BD Biosciences, catalog number: 557922)
 - b. Mouse Anti-human CD3-V450 (BD Biosciences, catalog number: 560365)
 - c. Mouse Anti-human CD25-APC (BD Biosciences, catalog number: 340939)
 - d. Mouse Anti-human CD69-FITC (BD Biosciences, catalog number: 555530)
 - e. Mouse Anti-human HLADR-PE (BD Biosciences, catalog number: 347367)
 - f. Mouse Anti-human CD279-PeCy7 (BD Biosciences, catalog number: 561272)
Note: Isotype controls are used at the same concentration as the specific antibody. So, dilutions have to be determined according to the concentration of the matched antibody.
 - g. PE-Cy™ 7 Mouse IgG₁, κ Isotype Control (BD Biosciences, catalog number: 557646)
 - h. FITC Mouse IgG₁, κ Isotype Control (BD Biosciences, catalog number: 556649)
 - i. V450 Mouse IgG₁, κ Isotype Control (BD Biosciences, catalog number: 561504)
 - j. Alexa Fluor® 700 Mouse IgG₁, κ Isotype Control (BD Biosciences, catalog number: 557882)
 - k. APC-H7 Mouse IgG₁, κ Isotype Control (BD Biosciences, catalog number: 561427)
 - l. PE Mouse IgG_{2a}, κ Isotype Control (BD Biosciences, catalog number: 558595)
21. Dye-eFluor 506 (Invitrogen, catalog number: 65-0866-14)

22. DPBS (1x), Dulbecco's Phosphate-Buffered Saline (Corning Cellgro, catalog number: 21-031-CV), store at 4 °C for use after opening bottle
23. Dry Ice
24. HBSS (1x), Hanks Balanced Salt Solution (Thermo Fischer Scientific, Gibco™, catalog number: 141775-095), store at 4 °C for use after opening bottle
25. IMDM (1x) (Thermo Fischer Scientific, Gibco™, catalog number: 12440-053), store at 4 °C for use after opening bottle
26. Lymphocyte separation medium (Corning, catalog number: 25-072-CV), store at 4 °C for use after opening bottle
27. Penicillin/Streptomycin (Thermo Fischer Scientific, Gibco™, catalog number: 15140-122), store at -18 °C freezer before opening bottle and then store at 4 °C for subsequent use
28. RPMI 1640 (1x) with L-glutamine and 25 mM HEPES (Corning Cellgro, catalog number: 10-041-CV)
29. 0.05% Trypsin EDTA (1x) (Thermo Fischer Scientific, Gibco™, catalog number: 25300-120), store at -20 °C freezer before opening bottle and then store at 4 °C for subsequent use
30. RoboSep™ Buffer (Stemcell Technologies, catalog number: 20104)
31. Formalin
32. 10% RPMI (see Recipes)
33. 10% IMDM (see Recipes)

Equipment

1. Tissue culture flask vented caps, 70 ml (Falcon, catalog number: 353109)
2. Tissue culture flask vented caps, 250 ml (Falcon, catalog number: 353110)
3. Pipettes
4. Rocker
5. Incubator (Thermo Electron Corporation, NAPCO SERIES 8000 WJ, CO₂ Incubator)
6. Centrifuge: Sorvall Legend RT+ Centrifuge (Thermo Scientific)
7. Freezer
8. Luminometer (Promega GloMax Navigator, GM2000)
9. LN2 freezer
10. Magnets (Stem Cell Technologies, catalog number: 96290)
11. Light Microscope
12. 4 °C refrigerator
13. Flow cytometer (BD, model: LSR II)
14. Water Bath (Fisher Scientific, model: 2223)
15. Haemocytometer 0.100 mm deep (Hausser Scientific)

Software

1. FlowJo software (Tree Star)
2. BD FACSDiva™ software (BD Biosciences)
3. IUPMStats v1.0 Infection Frequency Calculator (Online) <http://silicianolab.johnshopkins.edu/>
4. Microsoft Excel

Procedure

Notes:

- a. *In order to get enough cells to keep the control for sets of experiment same, we leukophoresed control HIV-1 negative donors and also participants of the study, and froze those cells down at -140 °C.*
- b. *The TZA protocol may be adapted for fresh as well as frozen PBMCs.*
- c. *This procedure can be done in a BSL2+ lab following safety procedures involving wearing a closed lab coat and double gloves. The blood and processing should all be done in a bio-safety hood with proper airflow.*

A. Isolation of rCD4⁺ T cells from patients and controls

1. Collect blood from participants in EDTA tubes. This is usually done in the hospital by nurses and then sent over for processing.
2. Dilute the blood in DPBS (1:2). The total volume in a 40 ml tube should be 10 ml blood, 20 ml DPBS.
3. Then use 10 ml of lymphocyte separation medium or ficoll to separate the layers of cells in the blood. This is done by putting the pipette with ficoll at the bottom of the 50 ml tubes and slowly pushing the liquid in and a layer of clear ficoll will be at the bottom of the tube. The total volume of liquid will not become 50 ml in the tube.
4. Every reagent should be at room temperature before use.
5. Spin this in a centrifuge at 2,000 rpm (448 x g) for 20 min to get distinct PBMC layer which will appear in between the ficoll layer and the DPBS and serum mixture. RBC will appear at the bottom of the tube.
6. Collect the PBMCs layer and wash it again with DPBS spinning in a centrifuge at 1,000 rpm (112 x g) for 10 min.
7. Discard the supernatant and collect the pellet (PBMCs). At this point you may either carry on with the rest of the procedure or freeze your cells in a freezing media (10% DMSO in FBS) and store them at -140 °C for long term storage.
8. Count the cells recovered and then use resting CD4⁺ T cell Isolation kit to separate out the rCD4⁺ T cells from the PBMCs. It is always a good idea to not run less than 50 million cells at once since the yield in this kit is not high. It is a high purity but a low recovery kit. Also do not do

two 5 min separation for less than 100 million cells, rather do one-time separation through the magnet. The details of the separation are listed in the protocol that is sent with the separation kit.

9. Count the cells one more time before proceeding to the next step.

Notes:

- a. You will have to take some cells out and stain for resting T cells. This is important since it helps you determine whether the cells selected are purely resting T cells.
- b. If we are working with rCD4⁺ T cells, you can expect about 5 to 15 million rCD4⁺T cells from thawing about 100 million PBMCs for patients and about 20-25 million rCD4⁺ T cells from 100 million normal control PBMCs. This will not change irrespective of fresh or frozen cells.
- c. For thawing frozen cells, put the frozen cells dropwise slowly while moving the tube in circular motion in a 15 ml tube containing 10 ml of 10% IMDM. Then spin the cells down at 1,000 rpm (112 x g) for 10 min to wash it off from the freezing media.
- d. Follow the same Steps A1-A9 for cells from negative control participants. For every experiment you will need to do a control PBMCs with which the cells will be compared.

B. Assess the purity of resting T cells using Flow cytometry staining. (Figure 1)

1. First, transfer cells (2×10^5 to 2.5×10^5) into a tube and centrifuge at $400 \times g$ at $4^\circ C$ for 5 min and remove the supernatant.
2. Perform the viability staining: resuspend the cell pellets with 250 μl of DPBS and 1 μl of Viability Dye-eFluor506. Incubate for 30 min at $4^\circ C$.
3. Wash the cells with DPBS and centrifuge the plate at $400 \times g$ at $4^\circ C$ for 5 min and remove the supernatant.
4. Resuspend the cell pellets first in 100 μl of DPBS and then put this in a mixture of the antibodies in 5 ml round-bottom polystyrene tubes with 5 μl of each antibody listed in Materials and Reagents and incubate for 30 min at $4^\circ C$.
5. Wash the cells at $400 \times g$ at $4^\circ C$ for 10 min and remove the supernatant. Do this two times.
6. Fix the cells in 1% Formalin to run your flow. You can run your flow in plates or tubes, but we prefer tubes.
7. First gate on singlet population and them on lymphocytes. Follow this up by gating on the live population. In the live population of cells look specifically at the CD4⁺/CD3⁺ cells as these are the T cells.
8. Then the rest of the gating is from the CD4⁺/CD3⁺ cells. Gate them for CD69, HLA-DR and CD25, which are all activation markers. PD1 is used for gating by us for determining cell death signal but it is not important to use if your follow up experiments do not require it.

Note: You can choose antibodies of your choice for activation marker but we used CD-25, HLA-DR, CD-69 as activation markers. We also used PD1 as a secondary marker since activated cells have lower PD1. You will be looking for cells which are negative for CD-25, HLA-DR, CD-69. We gated on CD4 and CD3 positive cells since we were looking for resting T cells. 95 % or more purity if good

for the resting T cells.

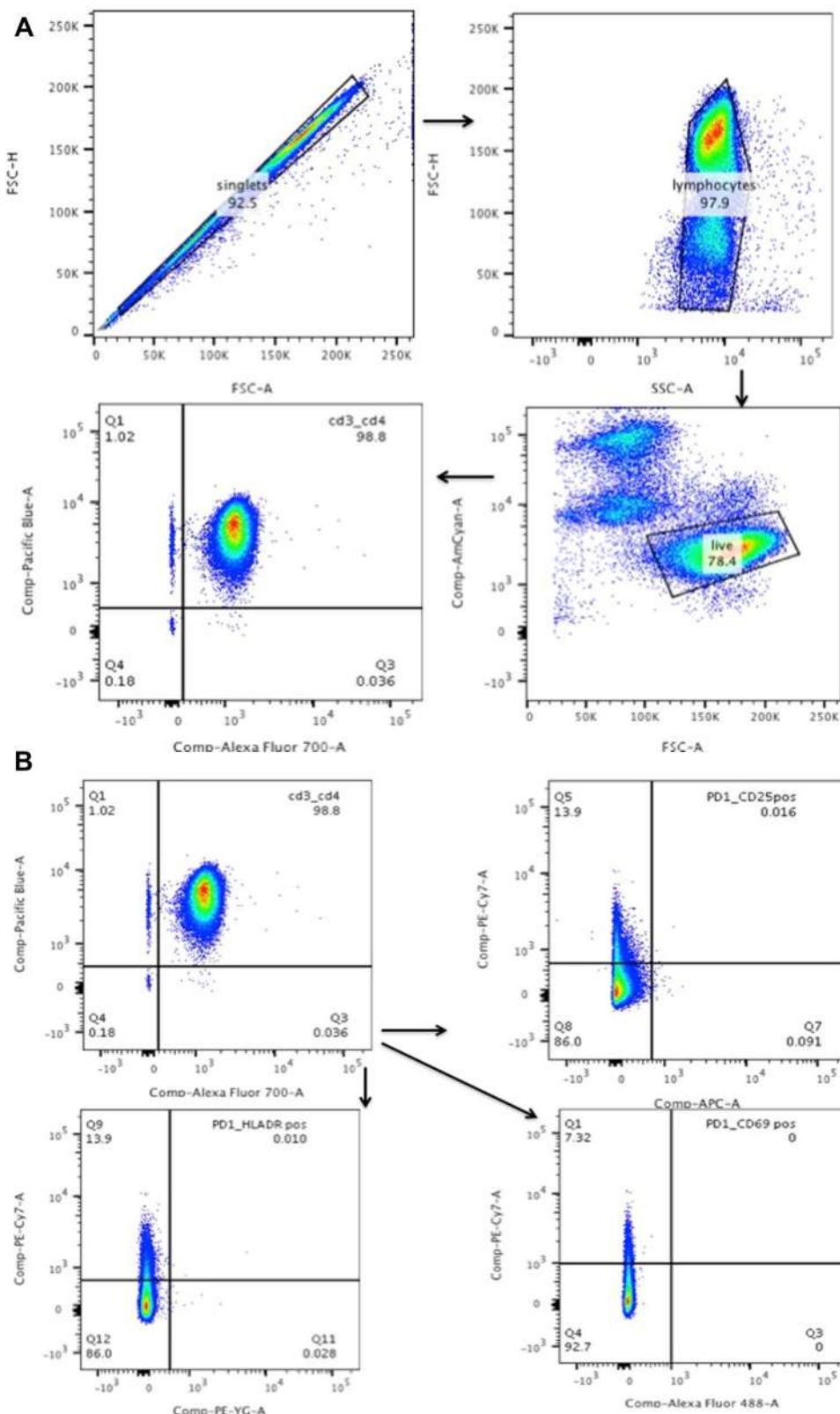


Figure 1. Purity of resting T cells: the resting T cells should be CD25/HLADR/CD69- CD3⁺/CD4⁺ cells. A. Gating on singlet population that is live and then to CD4⁺/CD3⁺ cells. B. Gating on CD3⁺/CD4⁺ cells to determine their activation status (CD25⁺/CD69⁺/HLADR⁺)

C. Activation of resting T cells

1. For the next step you will need to first stimulate the cells for activation. Use Anti-CD3/CD28 Dynabeads in a concentration of 12.5 μ l of the beads per million cells you put in the culture and they will stay in the culture together for 6 days till you do the next phase of the experiment.
2. Wash the beads before using them for activation. Put beads in a 5 ml polystyrene tube, put RoboSep™/EasySep™ buffer (1 ml) and put in magnet for 5 min. Then after decanting the liquid, resuspend the beads in 10% IMDM culture media exactly the amount you took out. For example, if you took out 100 μ l of beads for 8 million cells, you wash the beads, then resuspend it back in 100 μ l of 10% IMDM media and then out it in the final activation cocktail. (Figure 2).
3. The culture for stimulation is usually placed in 1.0 million cells/ml of media. Each well in a 24-well plate will usually have 2 million resting T cells, 2 ml of 10% IMDM media and 25 μ l of resuspended AntiCD3/CD28 Dynabead. After re-suspending the cells in media containing beads, put 300 nm of EFV per ml of the media to prevent cell-cell infection. Always prepare this fresh according to the amount of cell yield.
4. For best stimulation, the cells can be concentrated in smaller wells (48-well plates with 1 million/ml cells in one well) and then transferred to a 24-well plate next day. Plate 2 million/2 ml of cells per well in a 24-well plate (Figure 2).
5. After 24 h post activation, add 10 μ l of 10⁵ units/ml IL-2 and 10 μ l of 10⁵ units of IL-7 per million cells as the secondary signaling cytokines to maintain the cells and help them proliferate. Do not remove the Anti-CD3/CD28 Dynabeads.
6. The cultures need to be monitored every day and if there is overcrowding which is usually when the media becomes very yellow in color and the cell in each well is more than 4 million cells. Put more base media in the wells and split it into two wells in such cases.
7. The base media during splitting should always contain IL-2, IL-7 (on Day 2) and EFV along with 10% IMDM. No beads are required during addition of media to the cells in culture (Figure 2).
8. These cells stay in culture for a total of 6 days (Figure 2).

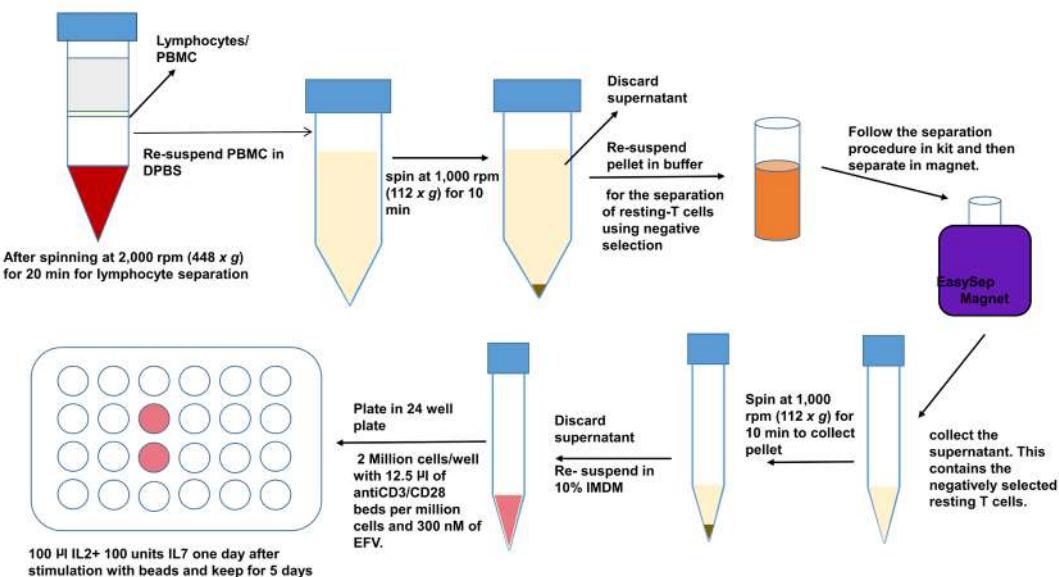


Figure 2. Processing latent cells for activation

D. Harvesting, plating and reading Beta-gal activity of TZM-bl cells

1. On Day 5 of the culture (one day before the T-cells are taken out of culture), seed 96-Well white PerkinElmer plates with 5×10^4 cells/well and rest of the plate with 3×10^4 cells/well of TZM-bl cells totaling 64-wells. Use 10% RPMI for media since TZM-bl cells grow in 10% RPMI. The total volume of media in each well should be 200 μ l. Keep this overnight so that cells adhere to the bottom of wells by next day.

Note: Don't maintain TZM-bl cells in culture for more than 3 weeks and split the cells every three days. When splitting, do not use more than 1 million/10 ml of 10% RPMI. TZM-bl cells grow in 10% RPMI in a 250 ml vented Falcon flask and need to be trypsinized when ready to split. When a frozen cell culture is started, make sure it is done in a 70 ml Falcon vented flask and then expanded over time.

2. On Day 6 from the start of culture, pull the activated T cells from the 24-well plates in 15 ml conical tubes and centrifuge at 1,000 rpm (112 x g) for 10 min to pellet the cells.

Note: You can save the supernatants for RNA if you want to compare the RNA yield of these activated latently infected cells. This can be frozen in -80 °C freezer. You will have to count the cells and wash them two more times with DPBS. You can also save 0.5 million cells for DNA quantification from each patient. For this, just count the cells, put them in Eppendorf tubes, spin them down at 1,000 rpm (112 x g) for 10 min and pellet the cells. Then directly just freeze these pellets in -80 °C freezer. This is used if you want to calculate the fraction of provirus induced for the particular participant.

3. With the rest of the activated T-cells, start to make a dilution series. Make a 4 fold dilution (1:3) starting from 1.25×10^5 cells/well in the highest dilution downward. (You can start as low as 6×10^4 cells/well on the top for this).

Note: If you are starting from 1.25×10^5 cells/well in the first row, each well in the top row (8 wells) should have 1.25×10^5 cells/well in 200 μ l of 10% RPMI. The second row will have $1.25 \times 10^5 / 4$ cells in all eight wells and so on downwards for five more dilutions. This should give you a total of 6 dilutions (Figure 3).

- After making the dilution series in 15 ml tubes, the media from the TZM-bl cell plates will have to be removed by aspiration before the patient cells are plated on it. Do not do it before you start to plate as it dries out the cells in the plate. The seventh row is usually not used and the eighth row is fresh media on the TZM-bl cells. (Figure 3).
 - All the dilutions will have to be in 10% RPMI.

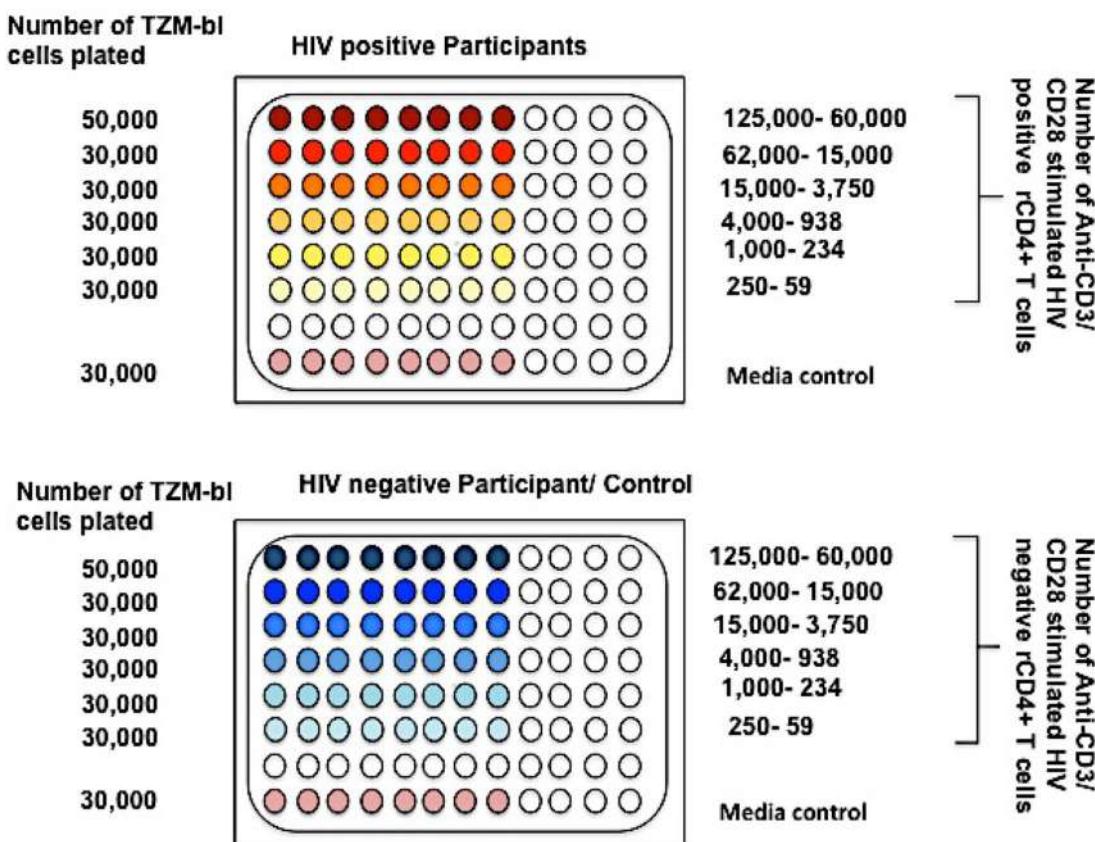


Figure 3. Plating cells for reading in 96 wells using TZM-bl cells

6. Keep this plate in a 37 °C incubator for 48 h after which it has to be read. This reading can be taken anywhere between 48 and 72 h.
 7. Take the plates out and aspirate the media out without disturbing the cells.
 8. Put back DPBS in the wells with multi-channel pipettes and lightly wash and take the washout and discard. Do this two times. Do not be very rigorous while washing as this could take the cells off from the plates.
 9. Put 100 µl of Promega Beta-Glo reagent on each well and incubate in the dark for 45 min-1 h.
Try to do all these steps in very little light. This reagent is extremely sensitive to light.

10. Then read in a Promega Glomax machine to get the light units following software prompt. This is an automated machine where you will have to select any of the cell-titer protocols, which will be able to read your plate. Then you can name and save the plate reads as excel files.

E. Analysis of readings and IUPM calculation

1. After the reading is taken, transport the Excel file out to analyze these results. First, calculate a mean (average) of the bottom media control row and deduct this value from all the other individual readings in the plate. This will be your blank. This should be done for both the control HIV-1 negative plate as well as the HIV-1 positive patient plate. This will also assure that you ideally have similar conditions for both the test and control cells.
2. In the control plate, which has the control HIV-1 negative T cells, calculate the average of each row with all the eight wells for each dilution. Do this for each dilution. Also, calculate the standard deviation for each dilution.
3. Add the average + 2 times the standard deviation (avg. + 2 SD) for each dilutions. This will give you a value for each dilution in the control well based on which you will calculate the positive wells in the test plate.
4. After you have the value of (avg. + 2 SD) for each dilution in the control plate, subtract it from each individual reading of wells for that particular dilution in the participant plate.
5. This will give you some wells with positive and some with negative values. The positive values can vary from 0/8 to 8/8.
6. Put these readings in the algorithm developed by Silicianos lab <http://silicianolab.johnshopkins.edu/> (Rosenbloom *et al.*, 2005) and calculate the Infectious unit per million cells or IUPM.
7. For DNA copies/million cells, use a standard DNA q-PCR (Cillo *et al.*, 2014) for integrated provirus in the cells and calculate the fraction of provirus that can be induced by the stimulation to form replication competent virus. This is calculated with the formula: fPVE = IUPM/DNA per million cells x 100

Data analysis

1. For the purity estimation of the resting T cells, samples are acquired on an LSR II flow cytometer using FACSDiVa™ software before putting the cells in culture for activation. Flow cytometry data are analyzed using FlowJo to gate, quantify, and analyze the resting T cell population.
2. For the IUPM calculations, the maximum likelihood estimate was applied to determine the infectious unit per million (IUPM) cells for the TZA assay using online software, available at <http://silicianolab.johnshopkins.edu/>, developed by Rosenbloom *et al.* (2015).

Notes

During the entire protocol, you have to make sure that you are doing exactly the same things for another set of cells which are HIV-1 negative or control cells. If you do not have your controls, your experiments will not work. You will have to make sure even the control cells are stimulated the same way as the patient cells so that ideally you have similar conditions for both the test and control cells.

Recipes

1. 10% RPMI

RPMI 1640

1x medium with 10% Animal Serum Complex and 1% Penicillin-streptomycin

Keep sterile and at room temperature during experiment and store at 4 °C

2. 10% IMDM

Mix 450 ml of IMDM w/L-glutamine with 50 ml FBS and 1% Penicillin-streptomycin (5 ml) from storage at -20 °C

This will make a final volume of 10% FBS in IMDM

This should always be stored at 4 °C for long term storage

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Competing interests

The authors declare no conflict of interest.

Ethics

Blood was collected and handled according to protocols approved by the University of Pittsburgh institutional review board and was pulled from the patient in the hospital by nurses. The participants both HIV-1 negative and positive were recruited from the Multicenter AIDS Cohort Study.

Written consent was obtained from all the participants and the study was explained to them in simple terms before proceeding with it.

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An Improved Bioassay to Study *Arabidopsis* Induced Systemic Resistance (ISR) Against Bacterial Pathogens and Insect Pests

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[Abstract] The plant immune system is essential for plants to perceive and defend against bacterial, fungal and insect pests and pathogens. Induced systemic resistance (ISR) is a systemic immune response that occurs upon root colonization by beneficial microbes. A well-studied model for ISR is the association of specific beneficial strains of *Pseudomonas* spp. with the reference plant *Arabidopsis thaliana*. Here, we describe a robust, increased throughput, bioassay to study ISR against the bacterial pathogen *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *Pseudomonas syringae* pv. *maculicola*) strain ES4326 and the herbivore *Trichoplusia ni* by inoculating *Pseudomonas simiae* strain WCS417 (formerly called *Pseudomonas fluorescens* WCS417) on *Arabidopsis* plants grown in Jiffy-7® peat pellets. While most commonly used for *Pseudomonas*-triggered ISR on *Arabidopsis*, this assay is effective for diverse rhizosphere bacterial strains, plant species, pathogens and herbivores.

Keywords: Induced Systemic Resistance (ISR), *Pseudomonas simiae*, *Arabidopsis thaliana*, *Pseudomonas syringae*, *Pseudomonas cannabina*, *Trichoplusia ni*

[Background] After plant infection or colonization by pathogenic or commensal microbes, a systemic defense response can ensue involving immunological “memory” or “priming” (Parker, 2009; Fu and Dong, 2013; Martinez-Medina *et al.*, 2016). Depending on the microbe and the associated plant organ, different systemic resistance programs are induced (Pieterse *et al.*, 2014). When roots encounter specific commensal microbes, induced systemic resistance (ISR) in distal root and shoot tissue is observed (Pieterse *et al.*, 1996; van Loon *et al.*, 1998). Although the genetic and hormonal signaling mechanisms deployed during the ISR response are partially understood (Verhagen *et al.*, 2004; Pieterse *et al.*, 2014; Pangesti *et al.*, 2016), many questions still remain unanswered, such as the mechanisms involved in root-microbiome communication or the identity of systemic signal(s) (Pieterse *et al.*, 2014; Haney *et al.*, 2018; Cecchini *et al.*, 2019).

The inoculation of *Arabidopsis thaliana* roots with *Pseudomonas simiae* WCS417 has been used as a model system for studying ISR against bacteria, fungi and herbivore invaders (Pieterse *et al.*, 1996;

van Loon *et al.*, 1998; Verhagen *et al.*, 2004; Haney *et al.*, 2015). Existing bioassays for *P. simiae*-*Arabidopsis* ISR make use of sterile sand-germinated *Arabidopsis* followed by seedling transplantation into a sand/soil mixture containing the rhizobacteria (Pieterse *et al.*, 1996; Pozo *et al.*, 2008; van Oosten *et al.*, 2008). Building on this method, we have recently developed ISR bioassays against the bacterial pathogen *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *Pseudomonas syringae* pv. *maculicola*) strain ES4326 (*Pma*) and the herbivore *Trichoplusia ni* (*T. ni*) using *Arabidopsis* plants germinated and grown in Jiffy-7® peat pellets. This is an effective system to robustly trigger and quantify ISR, primarily because peat pellets have a constant volume and low fluorescent pseudomonad content (Haney *et al.*, 2015 and 2018; Cecchini *et al.*, 2019). Moreover, avoiding the transplantation of seedlings reduces the experimental labor and stress to the seedlings, thereby improving assay efficiency. Here, we describe a step-by-step bioassay methodology for ISR experiments that can be potentially standardized across laboratories worldwide.

Materials and Reagents

1. 96-multiwell plates (Corning, Costar, catalog number: 2797)
2. Kimwipes (Fisher, catalog number: 06-666A)
3. 15 ml and 50 ml centrifuge tubes (MidSci™, catalog numbers: 15 ml-CT2715, 50 ml-CT2750)
4. Plastic domes (Hummert International, catalog number: 11-33480) and trays (Hummert International, catalog number: 11-33010)
5. 9 cm round Petri dishes (Fisher, catalog number: FB0875712)
6. 1.5 ml microfuge tubes (MidSci™, catalog number: AVSS1700)
7. Plastic pestles for 1.5 ml microfuge tubes (Fisher, catalog number: 12-141-364)
8. 3 or 5 mm metal beads (QIAGEN, catalog number: 69997 or 69989)
9. Trays for holding the Jiffy-7® peat pellets (Hummert International, catalog number: 11311000)
10. 1 ml-syringes without needle (BD Biosciences, catalog number: 309659)
11. Mesh bags (can be made by sewing a semi-oval out of a fine washable mesh material such as bridal veil; see Figure 3 for schematic; alternatively, perforated cellophane bread bags can be used)
12. Tightly closing plastic container (with screwcap lid)
13. Damp paper towel
14. Fine bristled paint brush
15. 96-well racked collection microtubes (optional, for 96-well tissueLyser format) (QIAGEN 19560)
16. *Arabidopsis thaliana* (L. Heyhn.) ecotype Columbia (Col-0)
17. *Pseudomonas cannabina* pv. *alcaliphila* (formerly called *P. syringae* pv. *maculicola*) ES4326 (Bull *et al.*, 2010) carrying an empty vector (*PmaDG3/Pma*) (Guttman and Greenberg, 2001) bacterial culture (stored in 15% glycerol at -80 °C)
18. *Trichoplusia ni* (eggs; Benzon Research or Natural Resources Canada)
19. *Pseudomonas simiae* strain WCS417 (formerly called *Pseudomonas fluorescens* WCS417)

20. Jiffy-7® peat pellets (Jiffy products, Canada, Hummert International, catalog number: 14-23700)
21. Glycerol (Sigma-Aldrich, catalog number: G7757)
22. K₂HPO₄ (Fisher, catalog number: P288)
23. MgSO₄ (Fisher, catalog number: BP213)
24. Proteose peptone No. 3 (BD Biosciences-US, catalog number: 211693)
25. Antibiotics
 - a. Rifampicin (GoldBio, catalog number: R-120)
 - b. Kanamycin (GoldBio, catalog number: K-120)
26. Agar (Fisher, catalog number: BP1423)
27. Sterile distilled and tap water
28. Bleach (Clorox Concentrated Germicidal Bleach)
29. Ethanol (Decon Labs Inc., catalog number: 2701)
30. Triton X-100 (Fisher, catalog number: BP151)
31. Tryptone (BD Biosciences-US, catalog number: 211705)
32. Sodium chloride (NaCl)
33. Yeast extract (BD Biosciences-US, catalog number: 212750)
34. 70% ethanol (see Recipes)
35. 25% bleach supplemented with 0.1% Triton X-100 (see Recipes)
36. 0.1% agar (see Recipes)
37. King's medium B (KB) (see Recipes)
38. Luria-Bertani medium (LB) (see Recipes)
39. 10 mM MgSO₄ (see Recipes)

Equipment

1. Laboratory glassware
2. 200 µl and 1 ml, and 20-200 µl multichannel micropipettes (Gilson)
3. Forceps (Grainger, catalog number: 4CR15)
4. 1 L beaker (Pyrex, 1000)
5. Handheld electric drill (DeWALT, model: DWD110)
6. Cork borer 4 mm diameter
7. Autoclave (Primus Sterilizer Co. Inc. 1317. C.R.N: 09415.1256)
8. Microcentrifuge (Eppendorf, model: 5415D)
9. Freezer (-80 °C) (Panasonic VIP Plus, model: MDF-V76VC-PA)
10. Plant Growth chamber at 20-23 °C with 12 h light/12 h dark (~75-100 µmol s⁻¹m⁻², cool white fluorescent; A1000 Conviron Growth Chamber with *Arabidopsis* Kit, or similar. Alternatively, a growth-room with ~135-145 µmol s⁻¹m⁻² -mix 50/50 of 400-watt sodium and metal halide bulbs or 75-100 µmol s⁻¹m⁻² cool white fluorescent bulbs can be used)
11. Shaker incubator at 28 °C (Barnstead Max, model: Q 5000)

12. Balance (Mettler Toledo, model: PB1501)
13. Spectrophotometer (Bio-Mini SHIMADZU)
14. Laminar flow hood (SterilGARD 3 Advance)
15. Incubator at 28 °C (VWR, 3020)
16. Analytical balance for weighing caterpillars
17. TissueLyser Beadmill (QIAGEN, catalog number: 85300) with 24 or 96 adapters (QIAGEN, catalog number: 69982 or 69984)

Procedure

- A. Hydration of Jiffy-7® peat pellets and *Arabidopsis* seed germination and growth (Figures 1A-1C)
 1. In 1.5 ml Eppendorf tubes, surface-sterilize 100-200 *Arabidopsis* seeds by washing with 70% ethanol for 2 min followed by 5 min with 25% bleach supplemented with 0.1% Triton X-100 and three washes in sterile water. Resuspend the seeds in 1-1.5 ml of a sterile solution of 0.1% agar (prepared in water and sterilized by autoclaving) using a pipette. Alternatively, chlorine gas sterilization can be used to eliminate endophytic bacteria, particularly to avoid microbial contamination for rhizosphere microbiome studies (Haney *et al.*, 2015).
 2. Prior to use, leave the seeds for 3-7 days at 4 °C to allow for their stratification.
 3. Prepare 8-12 Jiffy-7® pellets for each genotype per bacterial infection experiment (or 25-30 pellets per treatment for *T. ni* assays, see Data analysis) by placing them in a germination tray.
 4. Hydrate Jiffy-7® peat pellets (Jiffy Products International, Canada) by immersing the base of the pellet with tap water. Allow the pellets to stand in water for at least 1 h to allow them to hydrate.
 5. Once the pellets are hydrated, use a pair of clean/sterile forceps to gently even/level the top surface of the pellet.
 6. Sow 3-4 stratified seeds by pipetting them at the surface of each Jiffy-7® pellet and cover the trays with a plastic dome. Do not cover the seeds with soil.
 7. Transfer the trays to a growth chamber/room under 12 h day and 12 h night conditions at 23-20 °C day/night temperature regime, ~75-100 $\mu\text{mol s}^{-1}\text{m}^{-2}$ of cool white fluorescent light at rosette level, 50%-70% relative humidity. Alternatively, ~135-145 $\mu\text{mol s}^{-1}\text{m}^{-2}$ of mix 50/50 of sodium and metal halide light can be used.
- Note: Intensities of cool white fluorescence > 125 $\mu\text{mol s}^{-1}\text{m}^{-2}$ cause plants to bolt between 3 and 4 weeks and so should not be used.*
8. At 3-7 days post germination, carefully remove extra seedlings with clean/sterile forceps from the surface of the pellets leaving only a single seedling in each pellet (do not water seedlings during the first week after planting).
 9. Two to three times per week, bottom water the Jiffy pellets by flooding the tray with tap water and pouring the excess water off after 30-60 min.

B. *P. simiae* suspension preparation and seedling/pellets inoculation (Figures 1D-1F)

1. When seedlings are eight days old, streak *P. simiae* WCS417 strain from a frozen glycerol stock onto a King's B (KB) solid medium plate supplemented with Rifampicin (100 µg/ml). Allow it to grow for 24 h at 28 °C. Pick a single colony, streak it to a new plate and grow it for another 24 h.
2. Inoculate 15 ml of media supplemented with Rifampicin (100 µg/ml) (in a 50 ml Falcon tube) with a loop of confluent bacteria from the plate. Grow in a 28 °C shaker incubator for 16-24 h.
3. Centrifuge the culture at 3,000 x g for 5 min, discard the supernatant and resuspend the pellet in 15 ml of sterile 10 mM MgSO₄ solution. Repeat the centrifugation and finally resuspend the pellet in 15 ml 10 mM MgSO₄ by pipetting up and down.
4. Make a 1/10 or 1/20 dilution of the bacterial suspension (900 µl 10 mM MgSO₄ + 100 µl bacterial suspension for 1:10 or 950 µl 10 mM MgSO₄ + 50 µl bacterial suspension for 1:20 dilution) in a 1.5 ml tube and measure optical density at 600 nm (OD₆₀₀) in a spectrophotometer to estimate the OD₆₀₀ of the original *P. simiae* culture. (Divide the measured OD by the dilution factor).
5. In a clean (non-sterile) beaker, prepare at least 1 L of the *P. simiae* WCS417 inoculum by diluting the bacterial suspension culture in 10 mM MgSO₄ to a final OD₆₀₀ of 0.01.
6. Inoculate the ten-day-old seedlings with 6 ml of the bacterial solution per plant by gently pipetting on the top surface of the Jiffy-7® pellet, near the base of the seedling, taking care to avoid contact with the leaves or hypocotyl. Then, submerge the entire pellets for ~30 s in the same solution (using the 1 L beaker with the suspension) without allowing contact of the bacteria suspension with the seedling aerial tissues. One liter of bacterial solution can support the inoculation of at least 16 pellets. For the control/mock-treated plants follow the same steps but only using sterile MgSO₄.

Alternatively: If multiple strains are inoculated onto plants within the same flat, to avoid cross-contamination of bacterial strains between pellets, 2 ml of more concentrated inoculum (OD₆₀₀ = 0.02-0.1) can be applied to the Jiffy pellets. For these assays, the pellets should be fairly dry (generally not watered after the initial soaking) and 2 days should pass after inoculation before flood watering the plants. Using this method, multiple bacterial strains and buffer-treated controls can be grown within the same flat with no detectable cross-contamination between pellets. Additionally, significantly smaller starting volumes of inoculum can be prepared.

7. After these treatments, bottom water the plants as before for an additional 15-25 days (3.5-5 week-old plants) for *Pma* assays or 20 days (4.5 week-old plants) for *T. ni* assays.

Note: Different genotypes can be planted side by side in the same tray. Rotate different trays 2-3 times per week to minimize growth variation.

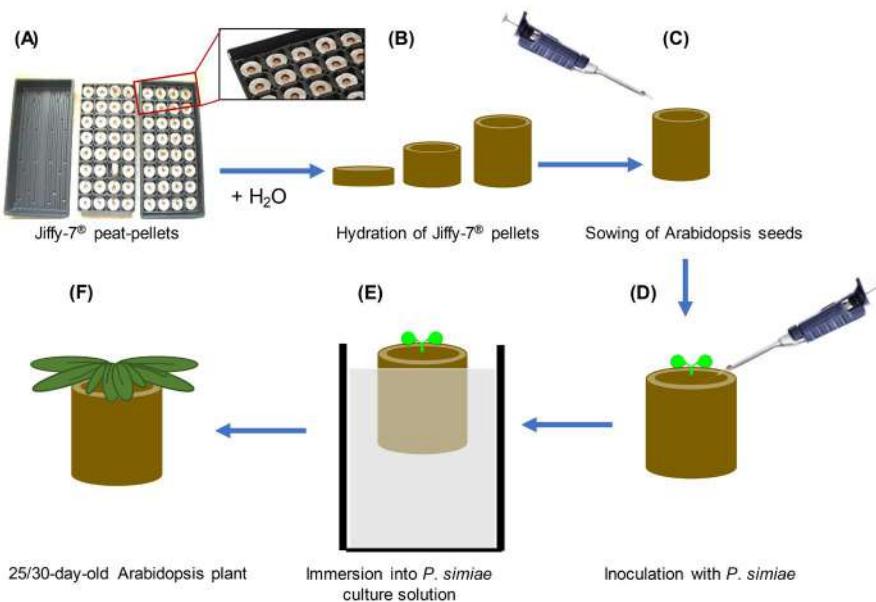


Figure 1. Schematic of *P. simiae* inoculation. A. Arrange Jiffy-7® peat pellets onto flats as shown. B. Flood the base of the trays with tap water for at least 60 min to allow the pellets to hydrate. C. Sow previously sterilized and stratified *Arabidopsis* seeds onto the surface of hydrated Jiffy® pellets. Water the pellets by flooding the base of the tray three times per week. D. Ten days after germination, inoculate 6 ml of *P. simiae* solution with OD₆₀₀ = 0.01 onto the surface of the pellet taking care to avoid contact with the seedling aerial tissues. E. Immerse for ~30 s the Jiffy® pellet into a 1 L solution of *P. simiae* of OD₆₀₀ = 0.01. One liter of bacterial solution can support inoculation of at least 16 pellets. F. Continue bottom-watering the pellets with tap water for a further 15 days (or 20 days for *T. ni* assay) until the plants are ready for plants infection with *Pma* or *T. ni*.

C. Infections and growth of *Pma* or *T. ni* to analyze the effects of *P. simiae* WCS417 ISR

To evaluate ISR against *Pma* (Figure 2)

Growing *Pma* and infection

1. Inoculate O/N cultures of *Pseudomonas cannabina* pv *alisalensis* carrying an empty vector (*Pma*) (Guttman and Greenberg, 2001) strain in KB-media supplemented with Kanamycin (50 µg/ml).
2. The next morning, dilute the cultures back in KB medium supplemented with Kanamycin (1:5 ml) and grow for additional 3-4 h.
3. Spin the bacteria down at 3,000 x g for 3 min and resuspend in sterile 10 mM MgSO₄. Repeat the wash an additional time.
4. Measure OD₆₀₀ of the suspension by diluting 1:20 as described above in section B, point 4. Dilute the culture to an OD₆₀₀ = 0.2 or 0.3 and dilute to a final OD₆₀₀ = 0.0002-0.0003 by performing three 10-fold serial dilutions in sterile 10 mM MgSO₄. The final volume should be 10 ml (~0.1 ml inoculum/leaf is required).

5. Use a 1 ml syringe without a needle pressed up against the abaxial side of the 5th and 6th *Arabidopsis* leaves to infiltrate the bacteria into the apoplast of an entire leaf. Infect at least two leaves on eight different plants. Use a felt-tipped marker to mark the petiole of infected leaves for identification leading to the sampling and quantification next steps (see below).
Note: Inoculate plants around 1 pm.
6. With a Kimwipe, dry the excess bacterial suspension from each infiltrated leaf. Let the leaves dry for 1 h until the liquid in the apoplast is no longer visible. Then, cover the plants with a plastic dome to increase the humidity and reduce the variability in bacterial growth. Return plants to the growth chamber for 2-3 days.

Quantifying bacterial growth in leaf tissues

7. Two or three days after *Pma* inoculation, excise 8 leaf discs from different plants per treatment from infected leaves (ISR-induced vs. mock) using a cork borer (4 mm diameter). Take the discs from approximately the same leaf position for all the samples, 3-5 mm from the leaf tip.
8. Using forceps, place each leaf disc in a 1.5-ml microfuge tube containing 200 µl of 10 mM MgSO₄.
9. Grind the samples using a small hand-held electric drill with a plastic pestle. Thoroughly macerate the tissue until pieces of intact leaf tissue are no longer visible to the eye (avoiding heating up the samples by performing repeated short bursts of ~2 s instead of grinding continuously for a long-time interval). We suggest grinding each sample for a similar duration to minimize variability during the experiment. Samples can also be ground with the plastic pestle by hand (without the drill).

Alternatively: Use a bead mill or TissueLyser to homogenize the samples. To use a TissueLyser, place the leaf disk into a 2 ml Eppendorf tube with a 5 mm metal bead and 100 µl water. Alternatively, a 96-well plate format can be used with 3 mm beads. Homogenize tissue for 2 min at 25 Hz.

10. Next, vortex the homogenate and remove 20 µl from each sample and dilute in 180 µl 10 mM MgSO₄ in 96-well multi-well plate. Use a multichannel pipette to repeat this process 4 times to have a serial 1:10 dilution series (10⁻¹ to 10⁻⁵) for each sample.
11. Plate 20 µl aliquots of the 1:10 serial dilutions (10⁻² to 10⁻⁵) on KB medium plates supplemented with Kanamycin (50 µg/ml of media) and allowed to dry onto the surface. Divide each KB plate with a marker such that at least eight samples/dilutions per plate can be plated.
Alternatively: By pouring media into rectangular plates, 10 µl can be spotted with a multichannel pipette, and all samples can be plated on a single plate (see Figure 2).
12. Incubate the plates at 28 °C for 2-3 days until colony-forming units (CFU) can be counted. Calculate the number of CFU per leaf disc by multiplying by the corresponding dilution factor (see Data analysis).

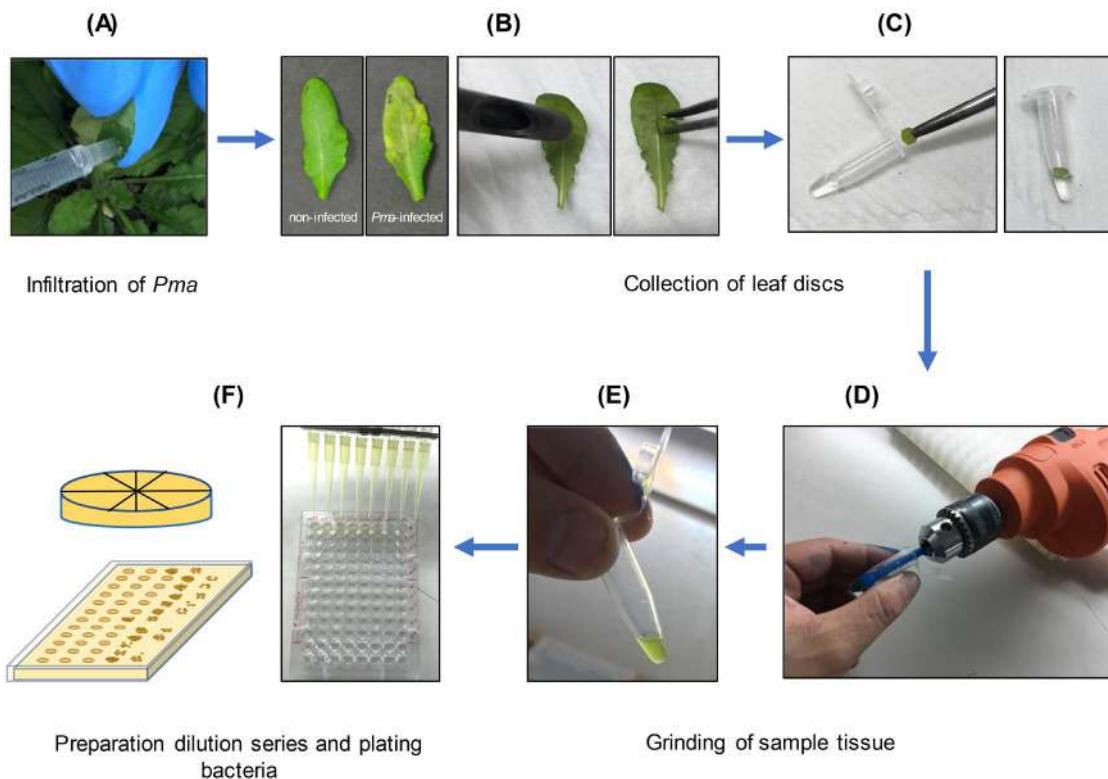


Figure 2. Images showing the key steps to evaluate ISR against *Pma* in *Arabidopsis* grown in Jiffy-7® peat pellets. A. Infiltrate the bacteria suspension into the abaxial surface of *Arabidopsis* leaves with a 1 ml-syringe without a needle (*Pma*, OD₆₀₀ = 0.0003) (Note: *Inoculate plants at ~1 p.m.*). B. Two-three days after *Pma* infection, excise leaf discs from the infiltrated leaves using a cork borer (4 mm diameter). C. Place each leaf disc in a 1.5-ml microfuge tube with 200 μ l of 10 mM MgSO₄ solution. D. Grind the samples using a plastic pestle mounted in a hand-held electric drill (shown) or a bead beater/TissueLyser (not shown). E. Grind the leaf discs until the tissue is thoroughly homogenized and no visible pieces of tissue remain (Note: *Avoid heating up the samples by performing ~2 s bursts*). F. Remove 20 μ l from each sample and dilute in 180 μ l 10 mM MgSO₄ in a multi-well plate and repeat this process to have a 1:10 dilution series (10⁻¹ to 10⁻⁵) for each sample. Plate 20 μ l aliquots of each dilution on KB medium plates or 10 μ l aliquots onto rectangular plates.

To evaluate ISR against *Trichoplusia ni* (Figure 3)

1. Incubate *T. ni* eggs (Benzon Research or Natural Resources Canada) at 30 °C for 36 h with 12-h light days. The photoperiod of the chamber should be in synch with that of the plants so the caterpillars are entrained with the same photoperiod.
2. Using a minimum of 25 plants per treatment at 4-5 weeks of age, randomly choose one newly hatched caterpillar larva from the batch. Using a small paint brush, place one larva at the center of the rosette of a Jiffy-7® pellet grown *Arabidopsis* rosette pretreated with *Pseudomonas* or buffer control.

3. Cover each pellet/plant with a mesh bag (Figure 3), return to a growth chamber, and allow the caterpillars to feed for 7 days. Plants should be at a density of no more than 30 per flat.
4. On the 7th day, remove the mesh bag and find the caterpillar on the plant. Weigh the larva with a precision balance to the nearest tenth of a milligram. The weight of newly hatched larvae is negligible; thus, the final caterpillar weight correlates with how much the caterpillar ate and host plant susceptibility (Cui et al., 2002).

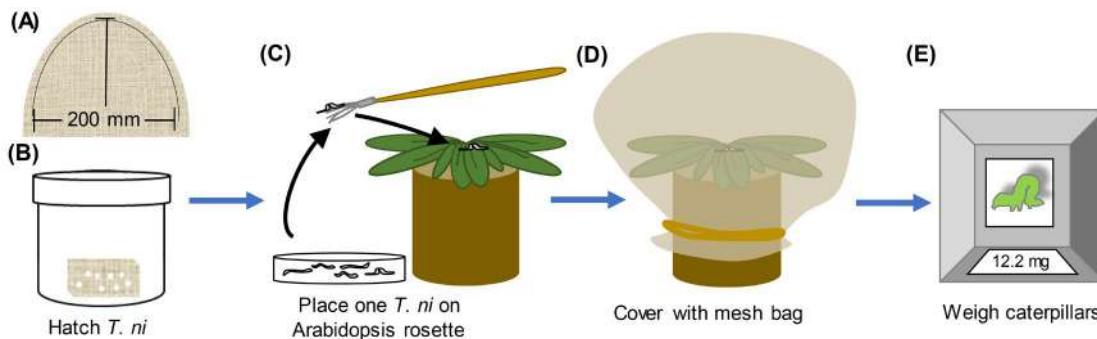


Figure 3. *T. ni* herbivory assay. A. Mesh bags can be made by cutting mesh (see materials) in a half-oval of ~200 x 200 mm and then using a sewing machine to sew two half-ovals together (dashed line). B. Place cheese cloth with *T. ni* eggs in a tightly sealing container with a damp paper towel at the bottom. Place the container in an incubator at 30 °C with the same light regime as the plants until the larvae hatch (~24–36 h). C. Using a fine paint brush, transfer a small number of newly hatch *T. ni* larvae to a Petri plate or other small container. Reseal the original container. Using the paint brush, place 1 larva on the center of the rosette of a 4–5 week-old *Arabidopsis* plant. D. Cover each plant with a mesh bag and secure at the base with a rubber band. Return the plants to the flats and return flats to the incubator. Allow caterpillars to feed for 1 week. E. One week later, find and weigh each individually (to the nearest 0.1 mg).

Data analysis

ISR-Pma data analysis

When the colonies are still small, count the CFUs for one of the dilutions (where 10–50 CFU can be counted and colonies are clearly distinct) for each disc from one leaf per plant taken and estimate the number of bacteria by multiplying with the corresponding dilution factor. Perform CFU counting for at least eight discs (12 discs provide greater statistical power and allows for increased confidence in smaller differences in bacterial growth). To increase confidence in possible differences in growth, replicate the entire experiment at least 3 independent times on different days and from different batches of inoculum. Use all the data collected from all independent experiments performed to calculate the average CFU per leaf disc plus/minus standard error. To determine if differences are statistically significant, perform analysis of variance (ANOVA) and a post-hoc test such Tukey's HSD test or Newman-Keuls (SNK) by using appropriate statistical software (Figure 4D and methods in

Cecchini *et al.*, 2019). If only two conditions are being compared, a Student's *t*-test can be used. Plot the data on a log₁₀ scale.

***ISR-T. ni* data analysis**

Weigh at least 25 larvae/plants per treatment, per experiment and repeat the experiment a minimum of 3 independent times. Use all the data collected from all independent experiments performed to calculate the average weight per larvae plus/minus standard error. To determine if differences are statistically significant, perform analysis of variance (ANOVA) and a post hoc test such Tukey's HSD test or Newman-Keuls (SNK) by using appropriate statistical software. If only two conditions are being compared, a Student's *t*-test can be used. If the experiments are done in different chambers or with insects from different sources, a significant replicate effect has been observed (Haney *et al.*, 2018). As a result, data can be normalized to the buffer-treated Col-0 control data from a single experiment prior to averaging the data from at least 3 independent experiments.

Notes

Using Jiffy-7® has allowed us to study interactions between diverse below and above ground commensals and pathogens. This setup has been used to study diverse rhizosphere bacteria that induce systemic resistance or susceptibility (Haney *et al.*, 2015 and 2018; Melnyk *et al.*, 2019). Additionally, it can be used to study below ground effects on diverse above ground pathogens including pathogenic *Pseudomonas* and *Xanthomonas* spp. (Haney *et al.*, 2018) and fungal pathogens such as *Botrytis*, and downy mildew.

Recipes

1. 70% ethanol
73.7 ml of 95% ethanol
Add distilled water up to 100 ml
2. 25% bleach supplemented with 0.1% Triton X-100
25 ml bleach and 100 µl of Triton X-100
Add distilled water to 100 ml
3. 0.1% agar
Dissolve 0.1 g agar in 100 ml sterile distilled water by autoclaving, swirl the solution while it cools
4. King's medium B (KB)
20 g of proteose peptone No. 3
10 g glycerol
1.5 g MgSO₄
1.2 g K₂HPO₄

For solid medium add 13 g agar

Add distilled water up to 1 L

Sterilize by autoclaving

5. Luria-Bertani medium (LB)

10 g tryptone

10 g sodium chloride (NaCl)

5 g yeast extract

Add distilled water up to 1 L

Sterilize by autoclaving

6. 10 mM MgSO₄

0.12 g of MgSO₄

Add distilled water to 100 ml

Sterilize by autoclaving

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Competing interests

The authors declare that they have no conflict of interests.

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Biofilm Formation Assay in *Pseudomonas syringae*

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[Abstract] *Pseudomonas syringae* is a model plant pathogen that infects more than 50 plant species worldwide, thus leading to significant yield loss. *Pseudomonas* biofilm always adheres to the surfaces of medical devices or host cells, thereby contributing to infection. Biofilm formation can be visualized on numerous matrixes, including coverslips, silicone tubes, polypropylene and polystyrene. Confocal laser scanning microscopy can be used to visualize and analyze biofilm structure. In this study, we modified and applied the current method of *P. aeruginosa* biofilm measurement to *P. syringae*, and developed a convenient protocol to visualize *P. syringae* biofilm formation using a borosilicate glass tube as the matrix coupled with crystal violet staining.

Keywords: Biofilm, *Pseudomonas syringae*, Plant pathogen, Borosilicate glass, Crystal violet, Visualization

[Background] Most *Pseudomonas* strains secrete exopolysaccharides, such as alginate, which is an important matrix molecule for biofilm formation (Hentzer et al., 2001; Nivens et al., 2001). Biofilm formed by the human pathogen *P. aeruginosa* plays important roles in its virulence and antibiotic resistance, and contributes to acute or chronic infections (Donlan and Costerton, 2002).

To date, various methods have been reported for biofilm characterization and quantification. Originally, biofilms were detected in microtiter plates made of polystyrene or polypropylene (O'Toole and Kolter, 1998; Merritt et al., 2005). During the growth of *P. aeruginosa* on a surface, the expression of genes involved in extracellular polysaccharide synthesis is induced (Davies et al., 1993; Davies et al., 1995), which promotes the adherence of cells to the surface. Crystal violet specifically stains the bacterial cells, and has been developed as a widely used dye for bacterial biofilm (George et al., 1998). Some recent studies have analyzed biofilm structure in a flow chamber coupled with confocal laser scanning microscopy (Sternberg and Tolker-Nielsen, 2006; Chua et al., 2016).

Biofilms formed by *P. syringae* strains have also been found in plant tissues (Osman et al., 1986; Fakhr et al., 1999; Preston et al., 2001). Alginate produced by *P. syringae* is an important polymer for *P. syringae* biofilm formation and contributes to its virulence and fitness, indicating its importance in plant-pathogen interaction (Preston et al., 2001; Engl et al., 2014). The formation of *P. syringae* and *P. fluorescens* biofilms can also be measured using crystal violet staining in microwell plates (Carezzano et al., 2017; Zhu et al., 2018; Patange et al., 2019).

In this study, we modified and applied the current method of *P. aeruginosa* biofilm measurement to *P.*

syringae (Kong *et al.*, 2015; Zhao *et al.*, 2016; Shao *et al.*, 2018). We present an economic, rapid and visual biofilm detection protocol that combines the use of borosilicate glass tubes and crystal violet staining methods, which have been efficiently used in our recent studies (Wang *et al.*, 2018; Wang *et al.*, 2019; Xie *et al.*, 2019) for visualizing the biofilm of the model plant pathogen *P. syringae*.

Materials and Reagents

1. 10 ml Borosilicate glass tube (ISOLAB, catalog number: 077.02.003)
2. 14 ml sterile tube (SPL Lifescience, catalog number: 40014)
3. Filter (PALL Lifesciences, catalog number: AP-4219)
4. Strains *P. syringae* pv. phaseolicola 1448A (*Psph*) (Xiao *et al.*, 2007) and *rhpS* deletion mutant ($\Delta rhpS$) (Xie *et al.*, 2019)
5. NaOH (UNI-CHEM, catalog number: 1310-73-2)
6. MgSO₄·7H₂O (Aladdin, catalog number: 10025-84-0)
7. K₂HPO₄ (Aladdin, catalog number: 7758-11-4)
8. BactoTM Proteose peptone No.3 (AOBOX, catalog number: 01-049)
9. Rifampin (Aladdin, catalog number: 13292-46-1)
10. Agar (MP Biomedicals, catalog number: 9002-18-0)
11. Crystal violet (Beijing Dingguo, catalog number: 548-62-9)
12. Glycerol (Beijing Bailingwei, catalog number: 262536)
13. 100% ethanol (Honeywell, catalog number: 32221-2.5L)
14. King's B (KB) (see Recipes)

Equipment

1. 1 ml pipette (Eppendorf, catalog number: 3123000063)
2. Benchtop shaking incubator (Labwit Scientific, model: ZWYR-240)
3. Constant temperature incubator (Labwit Scientific, model: ZXDP-B2120)
4. EvolutionTM 350 UV-Vis Spectrophotometer (Thermo Fisher Scientific, catalog number: 912A0959)
5. SynergyTM 2 Multi-Mode Microplate Reader (BioTek)
6. Test tube stand (ISOLAB, catalog number: 079.01.005)
7. -80 °C freezer (Thermo Scientific, catalog number: 5IDTSX)

Software

1. Microsoft Office Excel 2016 and GraphPad Prism 8.0.2.

Procedure

Notes:

1. Select wild-type *P. syringae* pv. *phaseolicola* 1448A (*Psph*) as the model strain (Xiao et al., 2007) and *rhpS* deletion mutant ($\Delta rhpS$) (Xie et al., 2019) as the test strain.
2. Perform the step-by-step protocol described in Figure 1.
3. Perform the entire procedure gently to avoid damaging the biofilm.
4. Collect the liquid crystal violet and ethanol waste liquor in specially labeled containers for professional disposal by trained staff, as per the safety regulations at City University of Hong Kong.

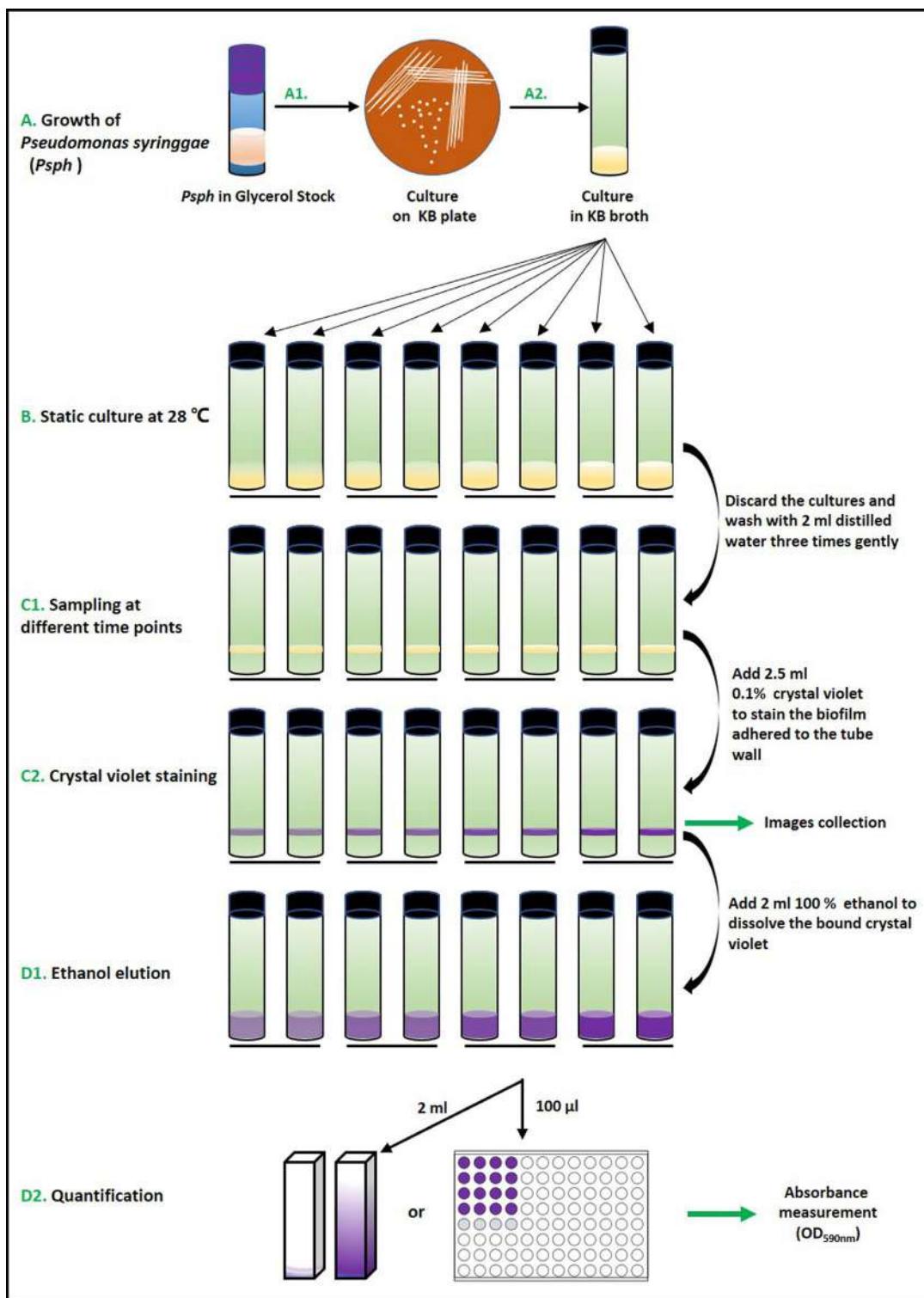


Figure 1. Schematic step-by-step protocol for visualizing *P. syringae* biofilm formation.
A. The *Psp* strain was activated on King's B (KB) plate and cultured in liquid medium. B. Then the cultures were inoculated at 1:500 dilutions into KB liquid medium and incubated statically to sampling points. C. Then stained the biofilm by using 0.1% crystal violet. D. Measure the biofilm production at OD_{590nm}. Two biological replicates were showed.

A. Bacterial growth

1. Collect a *Psp* colony from the glycerol stock culture frozen at -80 °C and inoculate on a King's B (KB) plate supplemented with rifampicin (25 µg/ml). Incubate the KB plate at 28 °C for 36 h in a constant temperature incubator.
2. Collect a single colony from the cultured plates and inoculate into a sterile 10 ml tube containing 2 ml KB liquid medium supplemented with rifampicin (25 µg/ml). Incubate the tube in a benchtop shaking incubator at 28 °C for 12 h with constant shaking at 220 rpm.

B. Biofilm formation

Inoculate the *Psp* culture (1:500 dilutions) into 16 sterile 10 ml borosilicate glass tubes containing 2 ml KB liquid medium (supplemented with rifampicin) and ensure consistent initial dose. Incubate the *Psp*-containing glass tubes at 28 °C in a constant temperature incubator without shaking.

C. Biofilm visualization

1. Harvest the biofilm samples at different time points. For *Psp* in our study, the biofilms were harvested at 24, 48, 72 and 96 h. Gently discard the planktonic cells with a 1 ml pipette and wash the tubes three times with sterile distilled water. Avoid damaging the biofilm formed on the tube wall.
2. Stain the biofilm forming bacteria with 2.5 ml 0.1% crystal violet for 20 min without shaking. Discard the dye and wash the tubes with sterile distilled water to remove the unbound dye. Dry the tubes and take photographs (Figure 2A).

D. Biofilm measurement

1. Elute the biofilm with 2 ml 100% ethanol and shake the tubes at 220 rpm for 20 min to ensure that the dye has dissolved completely. Take photographs (Figure 2B).
2. Measure the eluted samples at OD_{590nm} using a spectrophotometer (2 ml) or Synergy 2 Plate Reader (BioTek) (100 µl). If the sample concentrations are too high, diluted them before measuring. Use an equal volume of 95%-100% ethanol as the blank control. *Psp* biofilm formation is shown in Figure 2C. The $\Delta rhpS$ produces lower biofilm compared with *Psp* wild-type strain (Figure 3).

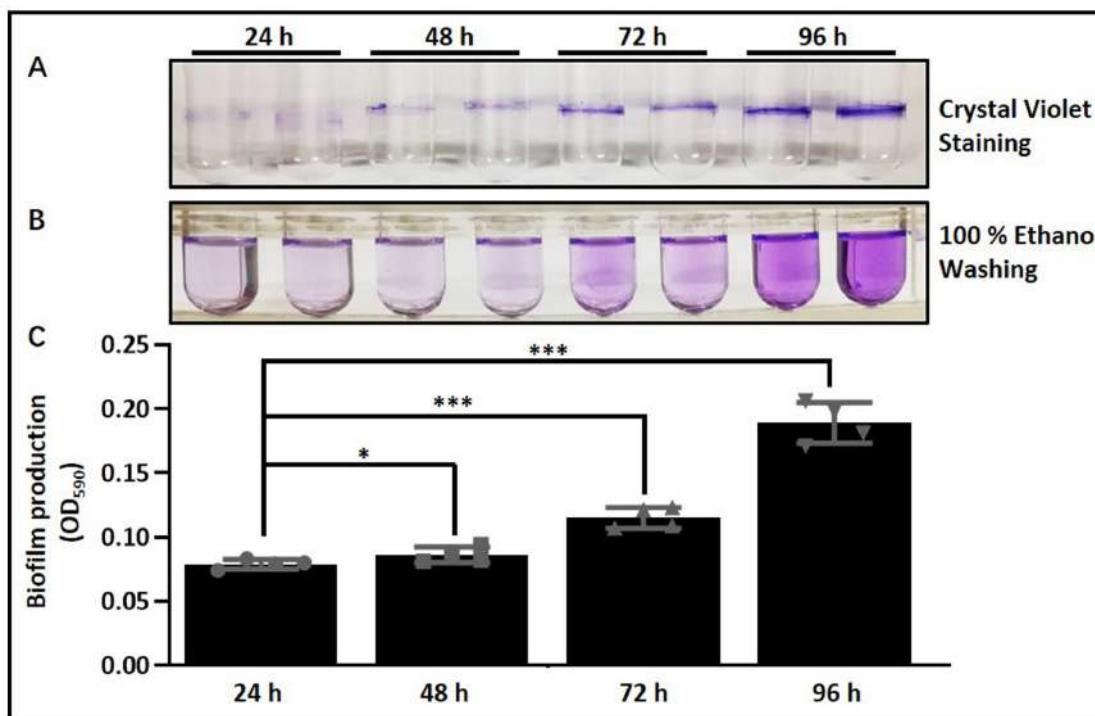


Figure 2. Visualization and quantification of biofilm in *PspH* wild-type strain. A. Biofilm samples were grown from 24 to 96 h. Biofilm adhered to borosilicate glass tubes at different time points were stained with crystal violet. B. The crystal violet bound to the biofilm on the wall of the tubes was eluted by ethanol. C. The elution samples were measured at OD_{590nm} by using spectrophotometer or Synergy 2 Plate Reader (BioTek). *PspH* wild-type strain produced more biofilm at 96 h than 24 h. * represents *P*-value < 0.05. *** represents *P*-value < 0.001. Error bars indicate S.D. among four biological replicates. Two biological replicates were shown.

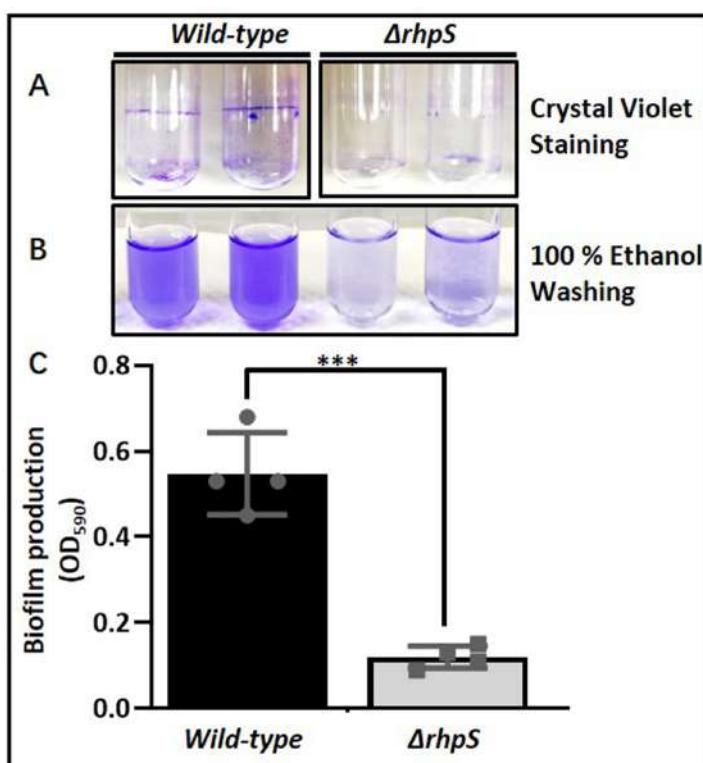


Figure 3. The $\Delta rhpS$ strain produced less biofilm than did that in *Psp*h wild-type strain.

A. Biofilm produced by the *Psp*h wild-type and the $\Delta rhpS$ strain were visualized using borosilicate glass tubes and stained with crystal violet at 96 h. B. The crystal violet bound to the biofilm on tube wall was eluted by ethanol. C. The *Psp*h wild-type strain produced more biofilm than the $\Delta rhpS$ strain (P -value = 0.000136). *** represents P -value < 0.001. Error bars indicate S.D. among four biological replicates. Two biological replicates were shown.

Data analysis

Student's *t*-tests were performed using Microsoft Office Excel 2016. Quantitative data (OD_{590nm}) were collected from four biological replicates in the figures and tables (Tables 1 and 2).

Table 1. Biofilm production of *Psp* wild-type strain presented in Figure 2C. Two-sample equal variance was calculated by the following one-tailed Student's *t*-test formula in Excel = TTEST (array1, array2, tails, type). Array 1 is the first data set. Array 2 is the second data set. Tails show the number of distribution tails (1 for the one-tailed distribution, 2 for two-tailed distribution). Type is the kind of *t*-test to perform (1 for paired, 2 for two-sample equal variance, and 3 for two-sample unequal variance. For example, we used TTEST (B2:E2, B3:E3, 1, 2), TTEST (B2:E2, B4:E4, 1, 2) and TTEST (B2:E2, B5:E5, 1, 2) for biofilm production at 48 h, 72 h, 96 h, compared to 24 h respectively. The *P*-values were showed in Excel H3, H4 and H5 respectively. * represents *P*-value < 0.05. ** represents *P*-value < 0.01. *** represents *P*-value < 0.001. Means and S.D. are shown in columns F and G. All experiments were repeated four times.

	A	B	C	D	E	F	G	H
1	Time(h)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean	S.D.	<i>P</i> -value
2	24	0.083	0.08	0.074	0.079	0.079	0.003741	
3	48	0.082	0.081	0.088	0.094	0.08625	0.006020	0.043390
4	72	0.123	0.121	0.109	0.107	0.115	0.008164	0.000101
5	96	0.171	0.181	0.198	0.206	0.189	0.015895	0.000005

Table 2. Biofilm production of the *Psp* wild-type and the $\Delta rhpS$ strain presented in Figure 3C. Two-sample equal variance was calculated by the following Two-tailed Student's *t*-test formula in Excel = TTEST (B2:E2, B3:E3, 2, 2). The *P*-values were showed in Column H3. * represents *P*-value < 0.05. ** represents *P*-value < 0.01. *** represents *P*-value < 0.001. Means and S.D. are shown in Column F and G. All experiments were repeated four times.

	A	B	C	D	E	F	G	H
1	Strains	Replicate 1	Replica te 2	Replicate 3	Replicate 4	Mean	S.D.	<i>P</i> -value
2	Wild-type	0.53	0.68	0.53	0.45	0.5475	0.096047	
3	$\Delta rhpS$	0.15	0.13	0.09	0.11	0.12	0.02582	0.000136

In sum, the results showed that the wild-type *Psp* strain produced more biofilm in 48 h (*P* < 0.05), 72 h (*P* < 0.001) and 96 h (*P* < 0.001) than at 24 h (Figure 2 and Table 1). Besides, the $\Delta rhpS$ strain produced less biofilm than *Psp* wild-type strain (*P* < 0.001) at the same time point (96 h) (Figure 3 and Table 2).

Recipes

1. King's B (KB) (King *et al.*, 1954)

Bacto™ Proteose peptone No.3 20.0 g/L

K₂HPO₄ 1.5 g/L

Glycerol 15 ml/L

Dissolve in 993.75 ml ddH₂O and adjust the pH to 7.2. Add 15 g/L agar for solidified media and autoclave

Dissolve 24.637 g MgSO₄·7H₂O in 100 ml ddH₂O and sterilize this stock solution (1 M) using sterile filter (0.45 µm)

Then, add 6.25 ml MgSO₄·7H₂O (1 M) (final concentration 1.5 g/L) into the autoclaved King's B medium when the temperature drops to 40 °C-50 °C

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This protocol was adapted from O'Toole *et al.* (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, 30: 295-304.

Competing interests

Conflict of interest statement: None declared.

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Fluorescence Microscopy Assay to Measure HIV-1 Capsid Uncoating Kinetics *in vitro*

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[Abstract] The stability of the HIV-1 capsid and the spatiotemporal control of its disassembly, a process called uncoating, need to be finely tuned for infection to proceed. Biochemical methods for measuring capsid lattice disassembly in bulk are unable to resolve intermediates in the uncoating reaction. We have developed a single-particle fluorescence microscopy method to follow the real-time uncoating kinetics of authentic HIV capsids *in vitro*. The assay utilizes immobilized viral particles that are permeabilized with the pore-former protein, and is designed to (1) detect the first defect of the capsid by the release of a solution phase marker (GFP) and (2) visualize the disassembly of the capsid over time by “painting” the capsid lattice with labeled cyclophilin A (CypA), a protein that binds weakly to the outside of the capsid. This novel assay allows the study of dynamic interactions of molecules with hundreds of individual capsids as well as to determine their effect on viral capsid stability, which provides a powerful tool for dissecting uncoating mechanisms and for the development of capsid-binding drugs.

Keywords: Retrovirus, HIV, Capsid stability assay, Uncoating, CypA, Viral particles, CA

[Background] The human immunodeficiency virus-1 (HIV-1) is a lentivirus that replicates in CD4-positive immune cells. HIV-1 copies its genomic RNA into DNA, which integrates into the host genome to establish a persistent infection within the host (Lusic and Siliciano, 2017). In the mature virion, the viral genome is enclosed within the capsid, a conical shell formed by the viral capsid protein (CA) organized into ~250 hexamers and exactly 12 pentamers (Briggs, *et al.*, 2003 and 2004). After fusion of the viral envelope with the host cell membrane, the capsid is released into the cytoplasm where it fulfills multiple essential functions, making capsid an attractive drug target. It not only protects the viral genome from degradation, but is also involved in influencing many viral post fusion events, including cytoplasmic transport, reverse transcription, nuclear import and integration site targeting (Ocwieja *et al.*, 2011; Schaller *et al.*, 2011; Lahaye *et al.*, 2013; Rasaiyaah *et al.*, 2013; Matreyek *et al.*, 2013; Jacques *et al.*, 2016; Dharan *et al.*, 2016; Sowd *et al.*, 2016; Burdick *et al.*, 2017). The viral capsid must ultimately disassemble in a gradual process termed uncoating to release the viral DNA. To regulate capsid stability and uncoating, HIV-1 capsid binds different sets of capsid-binding host molecules in the cytoplasm, at

the nuclear pore complex and inside the nucleus. For example, the peptidylprolyl isomerase cyclophilin A (CypA) binds to multiple CypA binding loops that are exposed on the outside of the capsid, which may stabilize the capsid during transport from the cell periphery to the nucleus. A roadblock to dissecting the order of events and precise effects of host factors and capsid-binding drugs on uncoating is the unavailability of methods to measure the kinetics of the uncoating reaction at the single-particle level. Most of the current biochemical methods to study HIV capsid stability, such as *in vitro* uncoating of isolated cores (Kotov *et al.*, 1999; Forshey *et al.*, 2002; Shah and Aiken, 2011), the fate of capsid assay (Stremlau *et al.*, 2006; Yang *et al.*, 2014), or cyclosporin A (CsA) washout assay (Perez-Caballero *et al.*, 2005; Hulme *et al.*, 2011; Hulme and Hope, 2014) rely on observing the average behavior of large numbers of viral cores, which obscures the identification of intermediates in the uncoating process. Fluorescence microscopy methods can resolve the uncoating of individual capsids in the cytoplasm (Campbell *et al.*, 2008; Pereira *et al.*, 2011; Ma *et al.*, 2016; Francis *et al.*, 2016; Mamede *et al.*, 2017; Francis and Melikyan, 2018), but complementary *in vitro* methods that allow higher throughput measurements and detailed kinetic studies under defined conditions are still missing.

We recently developed a novel fluorescence imaging method to follow the real-time uncoating kinetics of individual HIV capsids *in vitro* and applied this method to reveal how capsid disassembly is modulated by different host factors and small molecules (Mallery *et al.*, 2018; Marquez *et al.*, 2018). In our method, HIV-1 virus-like particles loaded with GFP as a content marker are immobilized on a coverslip for observation by total internal reflection (TIRF) microscopy. Upon permeabilization, the pool of GFP retained by the viral membrane is released while the pool of GFP trapped inside the capsid remains. Simultaneously, fluorescence-labeled CypA contained in the bulk solution can access and “paint” the capsid by binding to sites on its exterior surface. CypA binding is reversible, whereby the intensity of the CypA paint signal (*i.e.*, the number of CypA molecules bound at equilibrium) is proportional to the size of the CA lattice. At the concentrations used in this assay (up to 1 μ M, *i.e.*, about 10-fold lower than cytosolic concentrations), CypA binding has no measurable effect on capsid stability. The assay allows the measurement of the two processes involved in capsid uncoating for each particle in the field of view: (1) The sudden loss of the residual GFP signal pinpoints the precise time at which the first defect appears in the capsid, and (2) the gradual decay of the CypA paint signal reveals the kinetics of CA lattice disassembly thereafter. The method has several advantages: it studies authentic HIV capsids *in vitro*, utilizing small sample volumes in the low μ l range; hundreds of individual capsids are monitored in a single experiment and analyzed as single particles, allowing the identification of intermediates in the disassembly pathway that are averaged out in ensemble experiments; it allows screening of the effect of several molecules and proteins on capsid stability as well as the study of their binding kinetics. Our system has potential as a medium-throughput assay for screening compounds that target the stability of the HIV-1 capsid lattice. Furthermore, the approach can readily be applied to study capsid uncoating for other enveloped viruses.

Materials and Reagents

A. Viral particle preparation

1. T25 cell culture flask
2. Microwell plate
3. 10 cm² culture dishes (BD Biosciences, catalog number: 353803)
4. Poly-L-lysine-coated glass-bottom (175 µm thickness) 96-well plates (Greiner Sensoplate, Sigma, catalog number: M4187)
5. HEK-293T cells (ATCC, catalog number: CRL-3216)
6. Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Invitrogen, catalog number: 11965-092)
7. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F2442-500ML)
8. 1x PBS (Gibco, catalog number: 10010031)
9. 1x trypsin-EDTA (Gibco, catalog number: 15400054)
10. Plasmid, psPAX2 (NIH AIDS Reagent Program, catalog number: 11348)
11. Plasmid, pNL4.3-iGFP-ΔEnv (Hubner *et al.*, 2007; Aggarwal *et al.*, 2012)
12. Polyethylenimine (PEI Max) reagent (Polysciences, catalog number: 9002-98-6)
13. 0.9% w/v sodium chloride (Sigma-Aldrich, catalog number: S8776)
14. EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific, catalog number: 21338)
15. HEPES (Sigma-Aldrich, catalog number: H3375-250G)
16. NaCl (Chem Supply, catalog number: SA046-5KG)
17. Cell culture media (see Recipes)
18. HBS pH 7.5 and 7.0 (see Recipes)

B. AF568-CypA preparation

1. 0.22 µm syringe filter
2. Amicon-15 Ultra centrifugal filtration device (10k MWCO, Merck, UFC901024)
3. BL21 (DE3) *E. coli* cells
4. Plasmid, pETMCSI-hCypA (pBH964) (Ozawa *et al.*, 2004)
5. Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, catalog number: 23236)
6. Alexa-Fluor 568-C5-maleimide dye (Thermo Fisher Scientific, catalog number: A20341)
7. AF568-CypA (see Procedure)
8. Complete EDTA-free EASYpack, Protease Inhibitor Cocktail tablets (Roche, catalog number: 04693132001)
9. LB medium (MP Bio, catalog number: 113002041)
10. Isopropyl β-D-thiogalactopyranoside (IPTG)
11. Liquid nitrogen
12. Ampicillin powder (Thermo Scientific, catalog number: BP1760-25)
13. 1% v/v acetic acid diluted from glacial stock (Ajax Finechem, catalog number: AJA1-2.5LPL)

14. BSA (Bovogen Biologicals, catalog number: BSAS-NZ 0.1)
15. DTT (Sigma-Aldrich, catalog number: 43815-25G)
16. TCEP (Sigma-Aldrich, catalog number: C4706-2G)
17. HEPES (Sigma-Aldrich, catalog number: H3375-250G)
18. NaN₃ (Sigma-Aldrich, catalog number: S2002-500G)
19. NaCl (Chem Supply, catalog number: SA046-5KG)
20. Lysozyme (Sigma-Aldrich, catalog number: 62971-10G-F)
21. MOPS (Sigma-Aldrich, catalog number: M1254-1KG)
22. Tris (Ajax Finechem, catalog number: AJA2311-5KG)
23. Glycerol (Ajax Finechem, catalog number: AJA242-2.5LGL)
24. Lysis buffer (see Recipes)
25. AIEX buffer A (see Recipes)
26. AIEX buffer B (see Recipes)
27. CIEX buffer A (see Recipes)
28. CIEX buffer B (see Recipes)
29. CypA storage buffer (see Recipes)
30. AF568-CypA storage buffer (see Recipes)

C. Microfluidic device preparation

1. Plastic weigh-boat (Sigma, or equivalent)
2. Tubing Intramedic 0.043 in. (Becton Dickinson, catalog number: 427406)
3. Disposable needles, 1 mm OD (Becton Dickinson, or equivalent)
4. Blu tack (Bostik, or equivalent)
5. Round glass coverslips, 25 mm diameter (Paul Marienfeld GmbH & Co KG, catalog number: 0117650)
6. Biotinylated poly-L-lysine-g-poly-ethylene glycol (SuSoS AG, PLL-g-PEG-biotin, PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-biotin(20%))
7. Streptavidin (Life Technologies Australia, catalog number: 434301)
8. Sylgard184 silicone elastomer kit (Dow Corning)
9. Absolute ethanol (Ajax Finechem, catalog number: AJA214-2.5LGL)
10. 1 M NaOH (Ajax Finechem, catalog number: AJA482-500G)
11. Isopropyl alcohol (Thermo Scientific, catalog number: AJA214-2.5LGL)
12. Tris (Thermo Scientific, catalog number: AJA2311-5KG)
13. EDTA (Ajax Finechem, catalog number: AJA180-500G)
14. NaN₃ (Sigma-Aldrich, catalog number: S2002-500G)
15. Tween 20 (Sigma-Aldrich, catalog number: P5927-500ML)
16. BSA (Bovogen Biologicals, catalog number: BSAS-NZ 0.1)
17. Blocking buffer (see Recipes)

D. Imaging assay

1. Bacterial pore-forming protein for viral membrane permeabilization

Option 1: Recombinant cysteine-less (C459A) perfringolysin O (PFO) with N-terminal His-tag was supplied by Michael Parker.

The protocols for expression and purification of recombinant PFO C459A have been described previously (Shepard *et al.*, 1998; Wade *et al.*, 2015). Production and handling of PFO should be done in accordance with local occupational health and safety regulations for handling toxins. Store frozen in small aliquots at -80 °C. After thawing an aliquot, store at room temperature for up to two months. The protein loses activity when stored at 4 °C or during repeated freeze-thaw cycles.

Option 2: Alternatively, streptolysin O (SLO) (Sigma-Aldrich, catalog number: S5265) can be used for permeabilization of viral particles (unpublished data).

Handling of SLO should be done in accordance with local occupational health and safety regulations for handling toxins. Dissolve the protein in PBS containing 2 mM TCEP to give a concentration of ~8 µM, prepare small aliquots, freeze in liquid nitrogen and store frozen at -80 °C. Thaw a fresh aliquot on the day of use. Unlike PFO C459A, wild type SLO loses activity in solution as a result of oxidation (disulfide formation).

2. Protocatechuic acid (Sigma-Aldrich, catalog number: 37580-25G-F)
3. Protocatechuate-3,4-dioxygenase (Sigma-Aldrich, catalog number: P8279-25UN)
4. Trolox (Sigma-Aldrich, catalog number: 238813-1G)

E. General

1. Sharp blades (TechnoCut, or equivalent)
2. Serological pipettes, 5,10 and 25 ml (Corning, or equivalent)
3. Microcentrifuge tubes (Eppendorf, or equivalent)
4. Sterile 15- and 50-ml conical tubes (Falcon, or equivalent)
5. Sterile pipette tips (Eppendorf, or equivalent)
6. Disposable syringes, 1 and 3 ml (Becton Dickinson, or equivalent)
7. Milli-Q Water (from Millipore Milli-Q Integral 5 Water Purification System, or equivalent)

Equipment

A. Viral particle preparation

1. 10-ml super loop
2. Tissue culture incubator, humidity, temperature and CO₂ regulated (Thermo Fisher Scientific, model: 3110, or equivalent)
3. Biosafety cabinet (Thermo Fisher Scientific, model: 1323TS, or equivalent)
4. HiPrep 16/60 Sephadryl S-500 HR column (GE Healthcare, catalog number: 28-9356-06)

5. Fast protein liquid chromatography (FPLC) system including injector, one pump, UV-detector, fraction collector (GE Healthcare, ÄKTA pure, or equivalent)
6. 4 °C refrigerator
7. -20 °C freezer
8. -80 °C freezer

B. AF568-CypA preparation

1. 5 ml HiTrap Q HP column (GE Healthcare Life Science, catalog number: 17115401)
2. 5 ml HiTrap SP HP column (GE Healthcare Life Science, catalog number: 17115201)
3. Zeba desalting spin column (Thermo Fisher Scientific, catalog number: 89883)
4. CelluSep T1 regenerated tubular membrane MWCO 3,500 Da (CelluSep, catalog number: 501546)
5. Branson Sonifier 250 Probe Sonicator mounted with a tapered microtip

C. Microfluidic set-up and capsid uncoating imaging

1. TIRF Microscope, see specifications in Part IV
2. Magnetic chamber for 25 mm round coverslips (Chamlide chamber, model: CM-B25-1)
3. Syringe pump (New Era Pump System Inc., model: NE-1002X)
4. Mold (e.g., prepared by photolithography on a silicon wafer or machined from aluminum) for making microfluidic devices; each device contains 5 flow cell channels; channel dimension 1,100 µm x 800 µm x 40 µm (length x width x height)
5. Plasma cleaner (Harrick Plasma, catalog number: PDC-32G)
6. Ceramic coverslip holder
7. Vacuum desiccator
8. Harris Uni-Core 1.0 mm punch
9. Oven
10. Andor iXon 888 EMCCD cameras

D. General

1. Pipettes (Gilson, catalog number: F167700, or equivalent)
2. Refrigerated tabletop centrifuge (Thermo Fisher Scientific, model: 75004524, or equivalent)
3. Tabletop centrifuge (Eppendorf Centrifuge, model: 5417R, or equivalent)
4. NanoDrop 1000 spectrophotometer or equivalent machine that can read UV-vis spectra
5. Rotator (Benchmark Scientific, model: R5010, or equivalent)

Software

Note: The following software package can be used individually or in combination for image analysis.

1. FIJI image analysis software (Schindelin *et al.*, 2012; <https://imagej.net/Fiji/Downloads>)

2. Matlab (Mathworks, <https://www.mathworks.com/products/matlab.html>)
3. JIM Immobilized Microscopy analysis package (<https://github.com/lilbutsa/JIM-Immobilized-Microscopy-Suite>)

Procedure

Part I: HIV-1 viral particle preparation

A. Produce HIV-1 viral particles (2.5 days)

Note: The combination of plasmids used in this protocol results in the production of virus-like particles that lack envelope proteins and are non-infectious; procedures should be done in accordance with local biosafety regulations for producing virus-like particles.

1. In a 15 ml conical tube, prepare DNA solution for transfection by mixing 6.6 µg of pNL4.3-iGFP- Δ Env plasmid, 3.3 µg of psPAX2 plasmid, 60 µl of 1 mg/ml PEI max solution in a final volume of 500 µl of 0.9% (w/v) sodium chloride. Incubate the mixture for 30 min at room temperature to allow formation of DNA:PEI complexes.
2. Split the HEK-293T cell culture as follows:
 - a. Remove the culture medium and wash the cell monolayer with 1x PBS. Add 1x trypsin-EDTA (typically 1 ml for a T25 cell culture flask) and incubate for 5 min at 37 °C.
 - b. Stop the trypsin digestion by adding new culture media and transfer the cell suspension to a 15 ml conical tube.
 - c. Take a sample to count cells and determine the total number of cells in the tube. Centrifuge the cell suspension at 300 x g for 5 min at room temperature to pellet the cells and discard the supernatant. Resuspend the cells in the appropriate volume of fresh culture media to obtain a concentration of 7×10^6 cells/ml.
3. Gently add 1 ml of cell suspension to the DNA:PEI mix and incubate for 5 min at room temperature.
4. Plate the cells:DNA:PEI mixture drop by drop in a 10 cm² culture dish containing 6.5 ml of culture media. Slightly shake the dish to distribute the cells homogeneously. Incubate at 37 °C with 5% CO₂ for 48 h.
5. Collect the virus-containing supernatant in a 15 ml conical tube and centrifuge at 2,100 x g for 10 min at 4 °C to remove cellular debris.
6. Collect the supernatant and transfer it to a new conical tube. The final volume of the cleared virus-containing medium should be around 7 ml.

Note: To verify the presence of fluorescent HIV particles in the supernatant, dilute 1 in 100 in PBS and then spinoculate 200 µl of the virus preparation onto a poly-L-lysine-coated glass-bottom 96-well plate well at 1,200 x g for 60 min at 4 °C, and inspect for fluorescent dots with a 60x objective in a fluorescence microscope. The number of viral particles per surface area can be determined by counting the number of fluorescent dots in at least 4 fields of view. The particle concentration can

then be obtained by multiplying the number of particles per surface area with the surface area of the well (to obtain the number of particles per well) and then dividing this number by the volume of supernatant added to the well (0.2 ml).

B. Biotinylate the HIV-1 viral particles (2 h)

1. Dissolve ~1 mg of EZ-Link™ Sulfo-NHS-LC-LC-Biotin in 1 ml of HBS pH 7.5 in a microcentrifuge tube.
2. Add the biotinylation reagent to the virus-containing medium and incubate in the dark for 90 min at 4 °C with gentle rotation.

C. Purify the biotinylated HIV-1 viral particles by size exclusion chromatography (1 day)

1. Connect a HiPrep 16/60 Sephadryl S-500 HR size exclusion chromatography column to the FPLC system. All solutions used for FPLC should be passed through a filtration membrane with 0.2 µm pore size for removal of particulates and to degas the solutions.
2. Replace the storage solution (usually 20% ethanol) with 2 column volumes (CV) of purified water and then equilibrate the column with 2 column volumes (CV) of HBS pH 7.5. Monitor the absorption at 280 nm. Flow rate: 1 ml/min.

Note: This step should be started the day before collecting the viral particles as it takes ~8 h for the column to be equilibrated.

3. Load the biotinylated viral particles (~6.5 ml) onto the column using a 10-ml super loop. Monitor the absorption at 280 nm. Flow rate: 0.5 ml/min.
4. Elute the sample with HBS pH 7.5 and collect 1 ml fractions until a total of 1.5 CV is reached (~6 h). A representative elution profile is shown in Figure 1.
5. Combine the fractions corresponding to the first small peak that contain the viral particles (typically around fractions 34-39 or C10-D3 on a microwell plate).
6. Purified biotinylated viral particles can be used within 7 days if they are stored at 4 °C, or you can make 200-500 µl aliquots and store at -80 °C (no flash freezing in liquid nitrogen needed). Frozen samples can be thawed on ice for use.
7. Wash the column with 2 CV of purified water and then with storage solution.

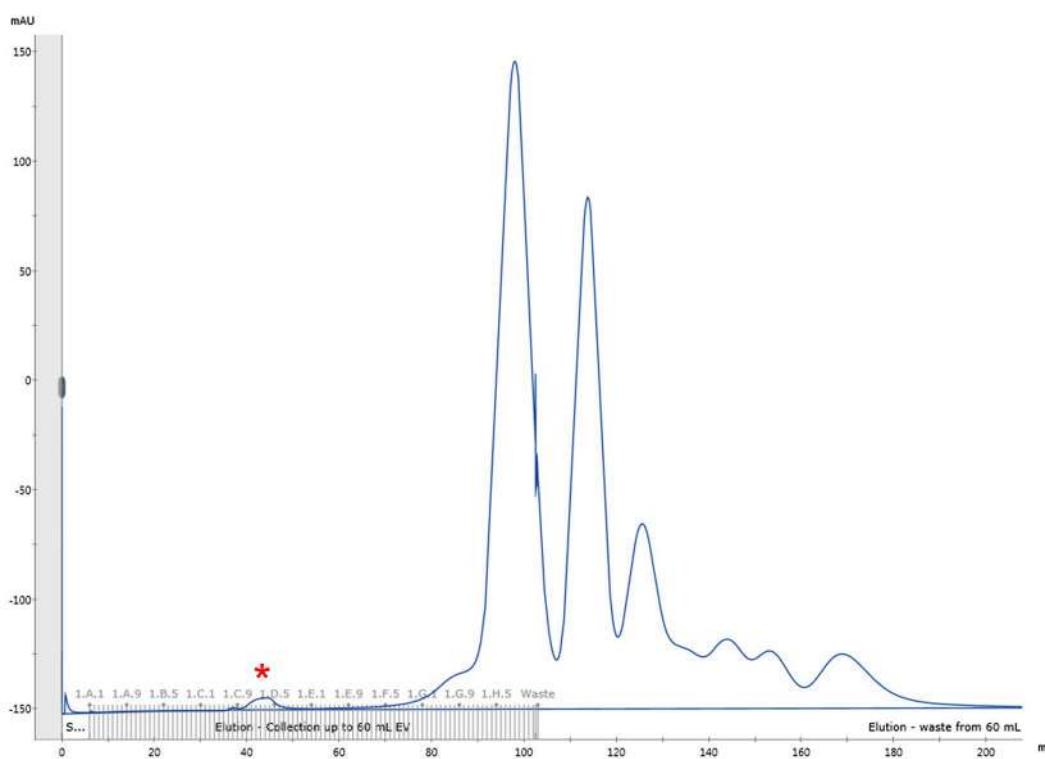


Figure 1. Representative elution profile of biotinylated HIV-1 viral particles by size exclusion chromatography. Chromatographic separation of HIV-1 viral particles from culture media proteins. The first small peak (fractions C10-D3, marked with a red asterisk) corresponds to the HIV-1 viral particles.

Part II: Preparation AF568-CypA for lattice painting

A. Express and purify human CypA (3-4 days)

1. Grow BL21 (DE3) *E. coli* cells expressing human CypA in a pET expression vector in LB medium supplemented with ampicillin (final 100 µg/ml) at 37 °C, 180 rpm, overnight as a starter culture.
2. Inoculate 500 ml of fresh LB medium supplemented with ampicillin (final 100 µg/ml) with 5 ml of starter culture.
3. Monitor the absorbance at 600 nm (OD₆₀₀). Induce protein expression by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the OD₆₀₀ reaches a value of 0.6. Allow protein expression to proceed for 3 h at 37 °C, 180 rpm.
4. Pellet the bacterial cells by centrifugation (6,000 × g, 20 min at 4 °C) and discard the supernatant.
5. Resuspend the cell pellet in a cold lysis buffer (typically use 15 ml of buffer per 500 ml of culture pellet) and lyse cells by sonication on ice (50% duty cycle, setting 3, 15 s on/off per cycle, 12 cycles).

Note: Cells can be lysed with any other sonicator or method for lysis of bacterial cells.

6. Centrifuge the lysate at 43,000 × g for 30 min at 4 °C, recover the supernatant and filter it through

a syringe filter ($0.22\text{ }\mu\text{m}$).

7. Purify the sample by subtractive anion exchange chromatography using $2 \times 5\text{ ml}$ HiTrap Q HP columns connected in tandem. First, wash the columns with 5 CVs of AIEX buffer A, followed by 5 CVs of AIEX buffer B and finally equilibrate with 5 CVs of AIEX buffer A at 5 ml/min . Load the sample and collect the flowthrough in 1 ml fractions. Keep eluting with extra $\sim 5\text{ ml}$ of AIEX buffer A to recover the entire flowthrough. Flow rate: 1 ml/min .
8. Check each flowthrough fraction on reducing SDS-PAGE and combine the fractions that contain CypA. Adjust pH to 5.8 with 1% v/v acetic acid using a pH probe to measure the pH. A representative elution profile is shown in Figures 2A and 2C.
9. Remove aggregates by centrifugation ($43,000 \times g$, 1 h , $4\text{ }^\circ\text{C}$) and collect the supernatant.
10. Purify CypA contained in the supernatant further using cation exchange chromatography using a 5 ml HiTrap SP HP column washed with 5 CVs of CIEX buffer A, followed by 5 CVs of CIEX buffer B, and finally equilibrated with 5 CVs of CIEX buffer A at 5 ml/min . Inject the sample onto the equilibrated column at 1 ml/min , then wash with 2 CVs of CIEX buffer A. Elute bound CypA with a $0\text{-}1\text{ M}$ linear gradient of NaCl ($0\%\text{-}100\%$ CIEX buffer B) over 20 CVs at 5 ml/min . The CypA peak will elute at $20\text{-}25\%$ CIEX buffer B. A representative elution profile is shown in Figures 2B and 2D.
11. Dialyze the fractions containing purified CypA against CypA storage buffer for $3 \times 4\text{ h}$ using dialysis tubing (e.g., CelluSep T1 regenerated membrane) with a molecular weight cut-off (MWCO) of $3,500\text{ Da}$.
12. Determine the concentration of protein using the Bradford assay with BSA as a standard.
13. If necessary, concentrate the protein using an Amicon-15 Ultra centrifugal filtration device (10k MWCO). The final recommended concentration should be $100\text{-}300\text{ }\mu\text{M}$ ($1.8\text{-}5.4\text{ mg/ml}$).
14. Use immediately or aliquot and flash freeze in liquid nitrogen for storage at $-80\text{ }^\circ\text{C}$.

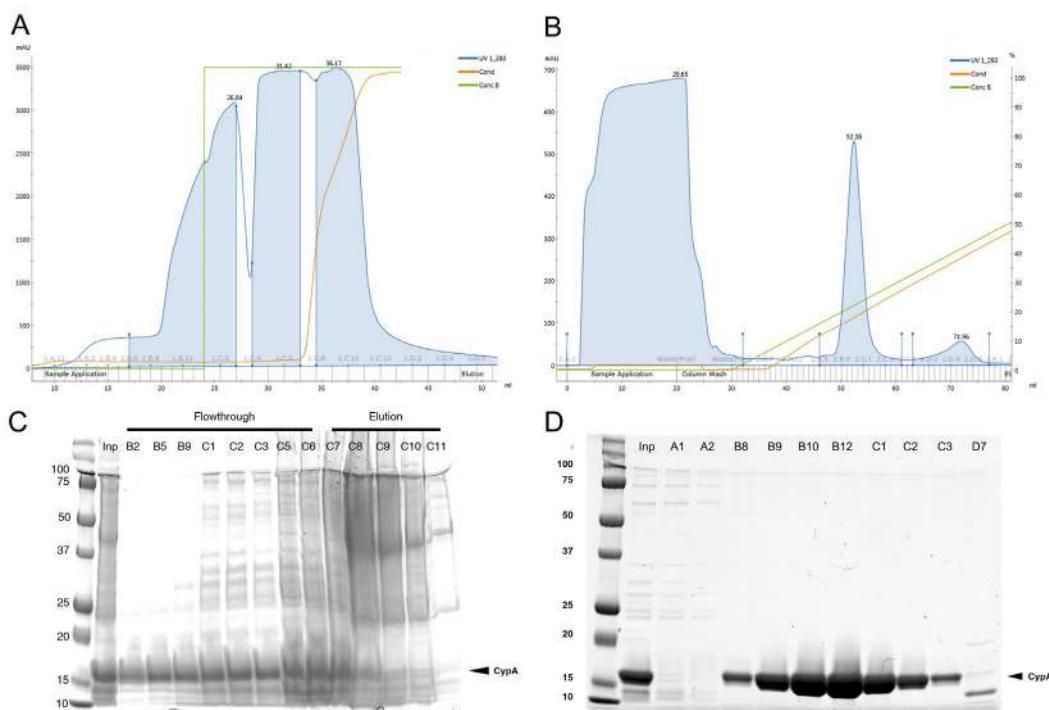


Figure 2. Purification of CypA by ion exchange chromatography. Elution profiles and corresponding SDS-PAGE analysis with Coomassie Blue staining of selected fractions from anion (A, C) and cation (B, D) exchange chromatography steps for CypA purification.

B. Fluorescence labeling of CypA (4 h)

1. Exchange the buffer of purified recombinant CypA using a Zeba desalting spin column equilibrated with PBS containing 0.1 mM TCEP to remove the DTT contained in the CypA storage buffer.
2. Mix purified CypA with a 4-fold molar excess of Alexa Fluor 568-C5-maleimide dye in PBS pH 7.4 and incubate for 10 min at room temperature. A typical reaction for labeling of 130 μ l of 100 μ M recombinant CypA requires 5 μ l of 10 mM Alexa Fluor 568-C5-maleimide.
3. Add DTT solution (1 M) to a final concentration of 10 mM to quench unconjugated dye.
4. Remove the free dye by exchanging the buffer using a Zeba desalting spin column equilibrated with AF568-CypA storage buffer.
5. Measure a UV-visible absorption spectrum of AF568-CypA using a NanoDrop 1000 or equivalent instrument to determine the absorption at 280 nm (A_{280}) and the absorption at wavelength of maximum dye absorbance (A_{dye} , ~578 nm for Alexa Fluor 568). Calculate the protein concentration (c_{CypA}) using the following formula: $c_{CypA} = (A_{280} - CF \times A_{dye})/\varepsilon_{CypA}$, where CF is a dye-specific correction factor (CF = 0.45 for Alexa Fluor 568) and ε_{CypA} is the molar extinction coefficient of CypA at 280 nm ($\varepsilon_{CypA} = 8730 \text{ M}^{-1}\text{cm}^{-1}$). Calculate the degree of labeling (DOL) using the following formula: $DOL = (A_{dye}/\varepsilon_{dye})/c_{CypA}$, where ε_{dye} is the molar extinction coefficient of the dye at its absorbance maximum ($\varepsilon_{dye} = 91300 \text{ M}^{-1}\text{cm}^{-1}$ for Alexa Fluor 568). AF568-CypA prepared by this protocol should have a degree of labeling close to 1 fluorophore.

per protein.

6. Prepare small aliquots, flash freeze in liquid nitrogen and store at -40 °C. Typically CypA-AF568 are stored in 2-4 µl at ~100 µM.

Part III: Microfluidic flow cells set-up

A. Prepare polydimethylsiloxane (PDMS) microfluidic devices (1.5 days)

1. Prepare 30 ml of the transparent polymer mixture (elastomer/curing agent ratio 10:1 w/v) in a plastic weigh-boat.
2. Mix thoroughly (avoid formation of bubbles) and then pour over the silicon microlithography wafer mold (Figure 3A).
3. Place the silicon mold containing the PDMS mix in a vacuum desiccator and degas under reduced pressure for 20 min to remove all air bubbles before curing.
4. Cure the PDMS overnight at 70 °C in an oven. The cured PDMS should be firm to the touch.
5. Carefully remove the PDMS devices from the mold by using a sharp blade (scalpel). Make sure you cut them into square pieces with dimensions that fit into the magnetic chamber used for imaging (see below).
6. Place the PDMS block onto a clean surface with the channels facing up and punch holes for inlets and outlets at opposite ends of each channel by pushing a Harris Uni-Core 1.0 mm punch through the PDMS device. Remove polymer plugs from the holes.
7. Clean the PDMS devices by immersion in water, isopropyl alcohol (100%) and water (10 min each step).
8. Dry PDMS devices with lint-free tissue and store in a clean container with the channels facing up.

B. Clean glass coverslips (3 h)

1. Place round glass coverslips in a coverslip holder and wash them by sonication in absolute ethanol for 30 min followed by sonication in 1 M NaOH for 30 min.
2. Rinse the coverslips by softly shaking the cover glass holder in a large beaker filled with ultrapure water. Repeat three times with fresh water each time. Handle carefully as the coverslips can stick together, fall out of the holder or break.
3. Wick away the excess of water from the coverslips in the holder with a lint-free tissue without touching the coverslip surfaces. Then place the holder with coverslips into a heated oven for at least 30 min for drying.
4. Use cleaned coverslips immediately or store them in a vacuum desiccator for use within a month.

C. Assemble the microfluidic flow cells (1-2 h)

Depending on the number of experiments you are planning to do, assemble one or more PDMS

devices. We recommend always preparing one extra device in case the glass coverslip breaks, the channels leak or something else happens.

1. Clean the channels of a PDMS device with isopropanol using a lint-free tissue. Let the surface dry.
2. Place a coverslip (in a ceramic holder) and a PDMS inside a plasma cleaner and treat them with an air plasma for 3 min. The channels should be facing up.
3. Carefully mount the PDMS device on the center of the coverslip with the channels facing the glass. Apply gentle, uniform pressure with your hand to ensure a uniform seal but without breaking the glass slide. To keep the coverslip clean, always place it on top of a lint-free tissue. The assembled device is shown in Figure 3B.
4. Place the assembled microfluidic device in an oven at 70 °C for 15 min to improve bonding between the glass and the PDMS.
5. Use immediately or store the devices in a clean container for use within 2 weeks.

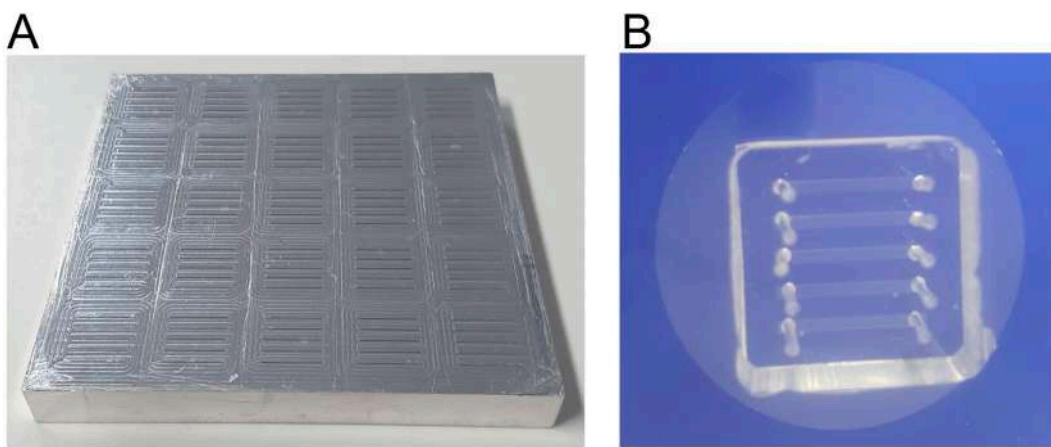


Figure 3. Microfluidic devices. A. Photo of the silicon microlithography wafer mold. B. Microfluidic device consisting of a PDMS block (with channels and holes for tubing) adhered to a glass coverslip.

D. Prepare inlet and outlet tubing (0.5 h)

1. For inlets, cut ~5-7 cm long pieces of tubing. Do not reuse inlet tubing.
2. For outlets, cut ~10-13 cm long pieces of tubing. Connect a blunted needle (1 mm OD) to one of the ends. To reuse them, rinse the tubing with distilled water using a syringe. Dry by drawing air through the tubing.

Part IV: Imaging assay

A. Microscope set-up

Images are acquired on a TIRF microscope. Our home-built system (designed by Philip Nicovich) is based on an ASI-RAMM frame (Applied Scientific Instrumentation) with a Nikon 100x CFI

Apochromat TIRF (1.49 NA) oil immersion objective. Lasers were incorporated using the NicoLase system (Nicovich *et al.*, 2017). Images were captured on two Andor iXon 888 EMCCD cameras (Andor Technology Ltd) and 300 mm tube lenses were used to give a field of view of 88.68 μm x 88.68 μm at Nyquist sampling frequency (86 nm per pixel). The TIRF angle was adjusted to obtain ~200 nm penetration depth.

Note: The relationship between TIRF angle and penetration depth and methods to empirically determine penetration depth are discussed in the following reference (Fish, 2009).

B. Capture viral particles onto the flow cell surface

1. With a P20 micropipette, gently inject a solution of biotinylated poly-L-lysine-g-poly-ethylene glycol (1 mg/ml) into one of the ports of each flow channel until the solution reaches the other port (~3 μl). Incubate for 30 min at room temperature.
2. Mount an assembled microfluidic device in a magnetic chamber and connect inlet and outlet tubing pieces to the opposite ports of each flow channel.
3. Place the inlet tubing of all channels into a microcentrifuge tube filled with ultrapure water (Figure 4A). Use removable adhesive to position the tube close to the device.

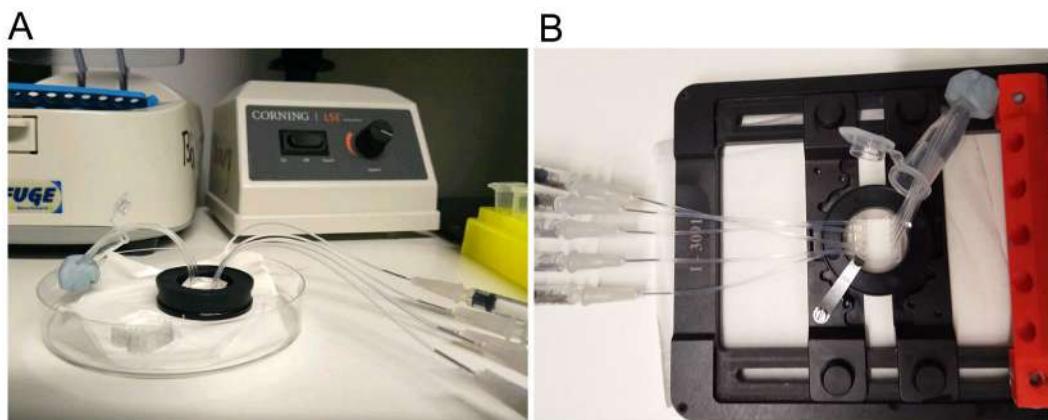


Figure 4. Setup for flow cell surface preparation. Picture of an assembled microfluidic device mounted on the magnetic holder in a (A) Petri dish or (B) on the microscope stage. Inlets (short tubing) are dipped into a microcentrifuge tube with HBS pH 7.5 while outlets (long tubing) are connected to a syringe.

4. Attach a 1 ml syringe to an outlet tubing and carefully rinse the channel by drawing distilled water through the tubing (~200 μl each). Repeat for all channels. You can use the same syringe for all outlets (switching from one to the next) or connect a separate syringe to each outlet for ease of use.
5. Remove the inlets from the water and remove the water from the channels by drawing air through them.

6. Draw a solution of 0.2 mg/ml streptavidin in blocking buffer into the channel using syringe connected to the outlet tubing (as in Step B4) and incubate for 15 min. A volume of 100 μ l of solution is sufficient to fill all five channels. From this step onwards it is very important to avoid letting air into the channels as exposure to air could degrade the modified surface, so always keep the inlets in a solution.
 7. Rinse the channel with HBS pH 7.5 to remove the streptavidin solution (~200 μ l per channel). Use modified flow channels on the day of preparation taking care to keep the channels filled with solution.
 8. Place the microfluidic device onto the microscope stage and connect the outlet tubing of the channel to be imaged to a syringe pump. Operate the syringe pump in "withdraw" mode to pull solutions through the channels.
- Note: The flow rates can vary from the values suggested below without damaging the modified surface (typically in the range between 5 and 100 μ l/min), whereby slow flow rates allow for more time for particle to diffuse and bind to the surface (e.g., in Step B9) while faster flow rates result in more efficient removal of non-specifically bound particles (e.g., in Step B11) and faster solution exchange rates.*
9. Inject purified biotinylated viral particles in HBS pH 7.5 through the channel at a flow rate of 25 μ l/min until a density of ~1,000 viral particles per field of view is obtained (typically 30-100 μ l).
 10. Place the objective at the center of the channel and focus on the surface. You should see the fluorescent viral particles bound to the surface as bright diffraction-limited spots.
 11. Wash-out the unbound viral particles with 50-100 μ l of HBS pH 7.5 at a flow rate of 65 μ l/min.

C. Perform capsid uncoating assay via painting with CypA

1. Prepare 40 μ l of CypA paint solution: HBS pH 7 containing PFO (200 nM), 0.5-1 μ M of AF568-CypA and an oxygen quenching system (2 mM trolox, 2.5 mM protocatechuic acid (PCA) and 0.25 U/ml protocatechuate-3,4-dioxygenase (PCD)) to reduce photobleaching during the dual color experiments. The pH of the imaging buffer is adjusted to pH 7 because PFO is considerably less efficient at higher pH (e.g., pH 7.5).
2. Initiate the assay by injecting 30 μ l of CypA paint solution at a flow rate of 65 μ l/min.
3. At the same time start acquiring TIRF images sequentially with 488 nm and 561 nm laser excitation. Example images are shown in Figure 5 and a typical image stack is shown in Videos 1 and 2.

Notes:

- a. *Laser power and exposure time should be chosen to obtain a signal-to-noise ratio that is sufficient to detect the level of GFP trapped inside the capsid (see below) while minimizing the energy that the sample is exposed to. High laser powers and long exposure times can lead to photochemically induced damage that affect the uncoating kinetics. On our system we typically use a power density of ~1 W cm⁻² (measured at the objective with the laser*

beam normal to the surface of the coverslip) and an exposure time of 20 ms. These settings avoid saturating the CCD camera in the GFP channel and result in a single molecule photobleaching rate of 0.02 s^{-1} (half-life of 35 s) in the AF568-CypA channel.

- b. The frame rate and total imaging time depend on the experimental question; we typically record capsid opening traces by acquiring 800 frames, e.g., at a frame rate of 1 frame/s (total imaging time of 13.3 min) for short experiments or at a frame rate of 1 frame/6 s (total imaging time of 80 min).

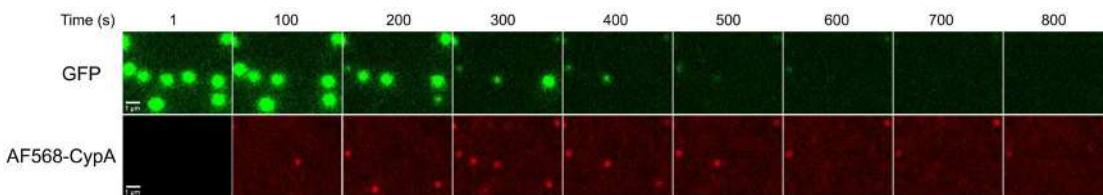
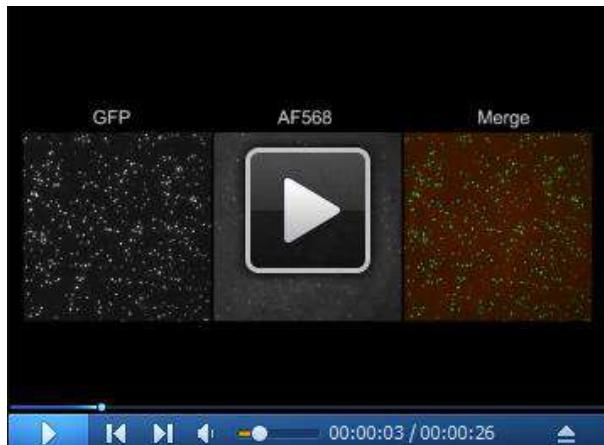
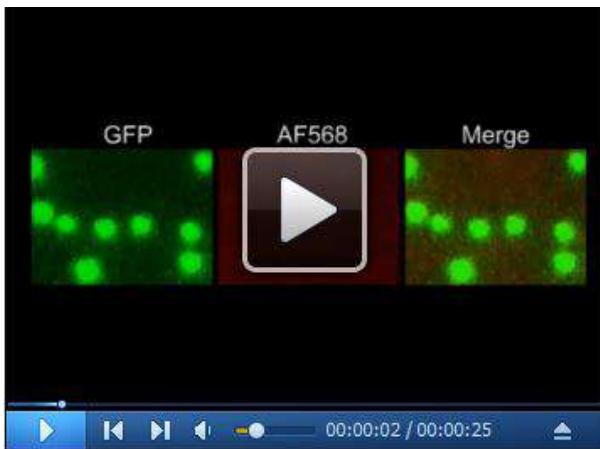


Figure 5. Capsid uncoating assay via painting with CypA. Snapshot images from a time series of viral particles recorded in the GFP channel (content marker, top) and the AF568 channel (CypA paint, bottom) at 1 frame per second. Snapshots correspond to indicated time points from the experiment shown in Video 2. Scale bars = 1 μm .



Video 1. Capsid uncoating assay via painting with CypA. Image stack of the GFP channel (content marker), AF568 channel (CypA paint), and merge of both videos of a typical capsid uncoating assay via painting with CypA (complete field of view, 1,024 x 1,024 pixels). Images were acquired at 1 frame per second.



Video 2. Capsid uncoating assay via painting with CypA. Image stack of the GFP channel (content marker), AF568 channel (CypA paint), and merge of both videos of a typical capsid uncoating assay via painting with CypA (80 x 60 pixel region of the field of view). Images were acquired at 1 frame per second.

4. Repeat this procedure in the remaining flow channels of the microfluidic device.
- D. Single molecule photobleaching of AF568-CypA
 1. Mount a clean coverslip in a magnetic chamber and add ~300 μ l of 0.02 mg/ml BSA to passivate it. Incubate for 2 min and remove.
 2. Wash with HBS 7 and add a solution of 10 nM of AF568-CypA in HBS pH 7. Incubate for 3–5 min.
 3. Remove unbound molecules by washing with HBS 7 and mount the chamber on the microscope stage for imaging.
 4. Acquire photobleaching movies in different fields of view with a 561 nm laser with the same laser power used during the uncoating assay and exposure times of 200 ms until most of the single molecules are photobleached (~150 frames).

Data analysis

Analysis is performed in three stages:

1. Creation of single particle traces using the Generate_Multi_Channel_Traces program in the JIM microscopy suite:
 - a. The two channels are aligned to each other and drift correction is calculated.
 - b. A subaverage of the start of the experiment is made to detect all particles.
 - c. Particles are detected using a threshold. Clusters of viral particles are excluded by setting the maximum eccentricity to 0.4. Background noise is excluded by setting the minimum number of pixels of a detected particle to 10.
 - d. The area around each particle is expanded by 4 pixels to ensure that all fluorescence is

captured. A region with a radius of 20 pixels further surrounding the particle is used to calculate the background fluorescence.

- e. The fluorescence intensity in each frame is calculated by taking the total fluorescence in each particle ROI minus the background fluorescence intensity.
2. Trace analysis
 - a. Identify the time and the height of GFP release steps (decrease in signal intensity) in each GFP trace (Böcking *et al.*, 2011). This can be automated using a step fitting algorithm (e.g., in MATLAB).
 - b. Sort the traces into different categories depending on the number of steps the signal has, according to the following classification criteria: (1) loss of entire GFP signal in one step (“leaky” particles, Figure 6A); (2) loss of GFP intensity in one large (permeabilization) and one small (capsid opening) step (“opening” particles, Figure 6B); (3) loss of the majority of the GFP signal in one step with residual GFP signal persisting for the rest of the experiment (“closed” particles, Figure 6C); (4) no permeabilization; (5) uninterpretable traces. Sorting can be automated by defining thresholds for the different intensity levels. The assignment to classes should be verified by visual inspection of traces.
 - c. To plot lattice disassembly of leaky capsids, align the individual binding traces from class 1 at the time of membrane permeabilization. To plot lattice disassembly of capsids that undergo uncoating during the experiment, align traces from class 3 at the time of core opening.

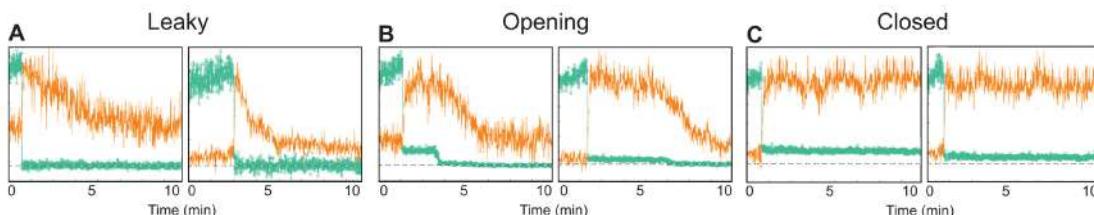


Figure 6. Capsid uncoating assay via painting with CypA traces. Example traces of CypA binding (vermillion) to individual leaky (A), opening (B) and closed (C) capsids that retain or do not retain GFP (green) after permeabilization of the viral envelope.

3. Single molecule photobleaching intensities are quantified using JIM.
 - a. Photobleaching traces are generated using the Generate_Single_Channel_Traces program.
 - b. Traces are stepfit to identify particles with single steps (representative of single molecules) using the Single_Molecule_Photobleaching method.
 - c. The step-heights of single step traces are fit to a gamma distribution and the modal intensity is taken as the single molecule intensity. A representative distribution of single step traces with fit is shown in Figure 7.
 - d. All traces from the CypA binding experiments are divided by this value to convert the traces from fluorescent intensity to the number of molecules.

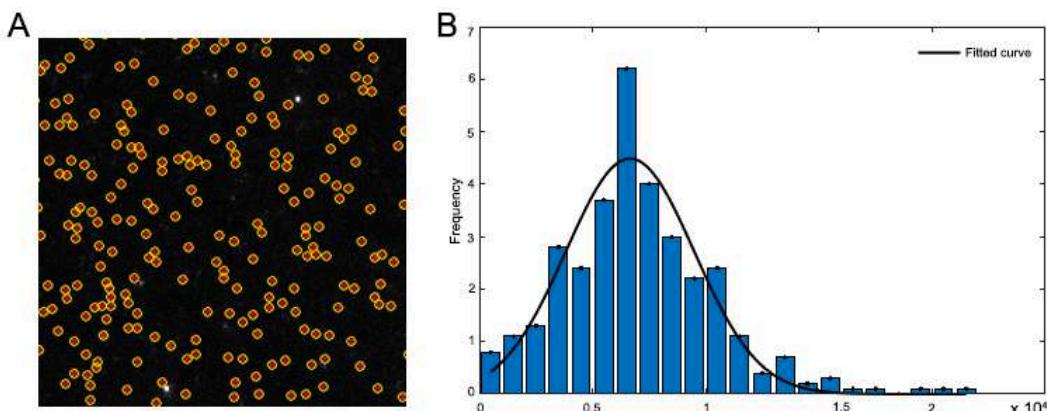


Figure 7. Single molecule photobleaching of AF568-CypA. A. TIRF image of AF568-CypA molecules adhered to the coverslip surface; particles detected by the image analysis software are marked with a red dot and a yellow circle. B. Representative distribution of step height from single-molecule photobleaching traces. The black line represents a Gaussian fit of the distribution.

Recipes

1. Cell culture media
Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS)
2. HBS
50 mM HEPES
100 mM NaCl
Adjust to pH 7.0 or 7.5
3. Lysis buffer
25 mM HEPES, pH 7.6
1 mM DTT
0.02% w/v NaN₃
1 mg/ml lysozyme
Supplemented with "Complete" protease inhibitor
4. AIEX buffer A
25 mM HEPES, pH 7.6
1 mM DTT
0.02% NaN₃
5. AIEX buffer B
AIEX buffer A
1.5 M NaCl
6. CIEX buffer A
25 mM sodium phosphate pH 5.8
1 mM DTT

- 0.02% NaN₃
- 7. CIEX buffer B
 - CIEX buffer A
 - 1 M NaCl
- 8. CypA storage buffer
 - 25 mM MOPS, pH 6.6
 - 1 mM DTT
 - 0.02% NaN₃
- 9. AF568-CypA storage buffer
 - 50 mM Tris, pH 8
 - 20% v/v glycerol
 - 1 mM DTT
- 10. Blocking buffer
 - 20 mM Tris pH 7.5
 - 2 mM EDTA
 - 50 mM NaCl
 - 0.03% NaN₃
 - 0.025% Tween 20
 - 0.2 mg/ml BSA

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Competing interests

There are no conflicts of interest or competing interest.

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Plaque Assay to Determine Invasion and Intercellular Dissemination of *Shigella flexneri* in TC7 Human Intestinal Epithelial Cells

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[Abstract] *Shigella flexneri* invades the epithelial cells lining the gut lumen and replicates intracellularly. The specialized Type III Secretion System (T3SS) and its effector proteins, encoded on a large virulence plasmid, assist the bacterium to gain access to the cytosol. Thereafter *Shigella* disseminates to neighboring cells in an epithelial layer without further extracellular steps. Host cell lysis occurs when these bacteria have extensively replicated in the target cell cytosol. Here we describe a simple method to qualitatively as well as quantitatively study the capacity of *Shigella* to invade and disseminate within an epithelium by assessing the number and size of plaques representing the dead cells in a monolayer of TC7 cells. This classical protocol follows a simple approach of infecting the monolayers of epithelial cell lines with *Shigella* and visualizing the dead cells as plaques formed against a stained background.

Keywords: Intracellular bacteria, Intestinal epithelial cells, Giemsa stain, *Shigella*, Invasion, Dissemination

[Background] Shigellosis is a gastrointestinal infection caused by one of four species of *Shigella* and resulting in about 600,000 deaths annually (Reference 15). Although the infections are reported worldwide, children aged 1-4 years in low- or middle-income countries bear most of the burden of the disease (Kotloff *et al.*, 2018). The bacterium infects the colonic epithelium where it gains access to the cytoplasm and replicates. This intracellular multiplication is followed by dissemination to adjacent cells and modulation of the immune response, eventually leading to cell death and destruction of the colonic mucosa (Ashida *et al.*, 2015). Gene mutations followed by *in vitro* assays such as the gentamicin protection assay or the plaque formation assay, have led to identification of various virulence factors of *Shigella* that assist the bacterium in invasion and dissemination. Most of these virulence factors are found on a giant virulence plasmid. Notably, the virulence plasmid encodes the Type 3 Secretion System (T3SS) as well as its effectors and regulators (Puher and Sansonetti, 2014). The T3SS system is necessary for invasion from the extracellular space. Once in the cytosol, *Shigella* moves via polarization of actin at one bacterial pole owing to the surface protein IcsA. These actin filaments (so-called “actin comets”) propel the bacteria into adjacent cells, which allows them to disseminate without further extracellular steps (Bernardini *et al.*, 1989). At the molecular level, dissemination is poorly understood, but it is thought to depend on the T3SS, similarly to invasion (Schroeder and Hilbi, 2008).

The plaque assay is a classical functional test to study two important steps in the *Shigella* infection

cycle, invasion events and bacterial dissemination (Oaks *et al.*, 1985). Intracellular replication and dissemination can alternatively be observed by fluorescence microscopy. However, this approach is more laborious and mostly qualitative, not quantitative. Many virulence factors have been identified using the plaque assay as the primary evaluation of the bacterial ability to disseminate to adjacent cells. After initial bacterial entry, the infected cells are covered with an agarose overlay containing gentamicin to eliminate the external bacteria resulting from unproductive spreading into the extracellular medium instead of adjacent cells, thereby ensuring that every plaque corresponds to a single invasion event at the beginning of the experiment. After 48-72 h of incubation, dead cells will have detached and are washed away. The intact cell layer is counterstained with Giemsa, resulting in a clear plaque that is visible against the colored background. The number of plaques gives an estimate of the invasive capacity of the bacteria under study. For example, invasive-deficient *Shigella* such as the *mxiD* mutant (Allaoui *et al.*, 1993) or the virulence plasmid cured strain BS176 (Sansonetti *et al.*, 1982) will not form any plaques. On the other hand, the size of plaques reflects the bacteria's ability to spread within an epithelium. For example, a strain that is fully impaired in dissemination such as *virG/icsA* mutants (Makino *et al.*, 1986; Bernardini *et al.*, 1989) will show no plaque formation. In contrast, strains that spread more slowly such as the *ipgD* mutant will lead to the formation of smaller plaques with respect to wild-type bacteria (Puhar *et al.*, 2013; Koestler *et al.*, 2018). This protocol can be used to compare the ability of different *Shigella* strains, including mutants, to disseminate from the initial site of infection. The protocol, however, is less suitable to assess the invasiveness of bacterial strains, which is readily achieved with the less time-consuming gentamicin protection assay. This protocol is an improvement of the classic plaque assay protocol described by Oaks *et al.* (1985). *Shigella* is an enteric pathogen and one of its primary targets are intestinal epithelial cells. While Oaks *et al.* (1985) and most of the literature thereafter used HeLa cells, an epithelial cell line originating from a cervix cancer sample, TC7 cells are intestinal epithelial cells. The detailed step-by-step nature of this protocol makes it highly reproducible and it can be applied to other *Shigella* species. Further, it can be modified to extend to other intracellular bacteria such as *Salmonella* or *Listeria*, or with other epithelial cell lines.

Materials and Reagents

1. Conical flask (Duran, Catalog number: 2121628)
2. Microfuge tubes (Eppendorf, Safe-Lock tubes, catalog numbers: 0030120086, 0030120094)
3. Centrifuge tubes (Sarstedt, catalog numbers: 62.554.502, 62.547.254)
4. Culture tube (TPP, catalog number: 91016)
5. Pipette tips (VWR, catalog numbers: 89041-404, 89041-412, 89041-400)
6. Serological pipettes (VWR, catalog numbers: 612-3702, 612-3700, 612-3698)
7. 6-well tissue culture test plates (TPP, catalog number: 92406)
8. Cell counter slides (Countess Cell Counting Chamber Slides, Thermo Scientific, catalog number: C10228)
9. TC7 cells (Chantret *et al.*, 1994)

10. *Shigella flexneri* M90T (Sansonetti *et al.*, 1982), can also be purchased from ATCC and available on request from the authors
11. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, catalog number: 21885-025)
12. Penicillin-Streptomycin solution (10,000 U/ml) (Gibco, catalog number: 15140122)
13. Minimum Essential Medium Non-Essential Amino Acids solution (100x) (Gibco, catalog number: 11140-035)
14. Dulbecco's Phosphate Buffered Saline (PBS) solution (Gibco, catalog number: 14190-144)
15. Fetal Bovine Serum (Gibco, catalog number: 10500056)
16. HEPES 1 M solution (Gibco, catalog number: 15630-080)
17. Trypsin-EDTA (0.05%), phenol red (Gibco, catalog number: 25300-054)
18. Trypan Blue Stain (0.4%) (Thermo Scientific, catalog number: T10282)
19. Gentamicin sulfate (Sigma, catalog number: G1264)
20. Agarose (VWR Life Science, catalog number: 35-1020)
21. Tryptic Soy Broth (TSB) ready to use powder (Merck, catalog number: 105459)
22. Tryptic Soy Agar (TSA) ready to use powder (Merck, catalog number: 105458)
23. Congo red (Sigma, catalog number: C6277)
24. Ethanol (VWR chemicals, catalog number: 20821.558)
25. Giemsa's azur eosin methylene blue solution (Merck, catalog number: 109204)
26. Growth medium (see Recipes)
27. Infection buffer (see Recipes)
28. Agarose overlay (see Recipes)
29. 5% (v/v) Giemsa solution (see Recipes)
30. Congo red solution (see Recipes)

Equipment

1. Biosafety cabinet (Thermo Scientific, HERAsafe KS 18)
2. Spectrophotometer (Amersham, Ultrospec 2100 pro)
3. Pipettman (VWR, Accurvette)
4. CO₂ Incubator (Thermo Scientific, Heracell VIOS 160i)
5. Benchtop centrifuge (VWR, Micro Star 17R)
6. Centrifuge (Eppendorf, 5810R with Rotor A-4-81 for plates)
7. Water bath (Grant, JBA 12)
8. Pipettes (Eppendorf, Research plus)
9. Inverted microscope (Motic, AE2000 Binocular)
10. Cell counter (Thermo Scientific, Countess II FL)
11. Imaging system (Bio-Rad, ChemiDoc XRS)
12. Microwave oven (Whirlpool, MD101)
13. Disposable scalpel (Swann-Morton, 0503)

14. Vacuum pump (VWR, Mini diaphragm vacuum pump VP 86)
15. Orbital shaker (Edmund Bühler, Swip SM25)

Software

1. Prism 7 (GraphPad, <https://www.graphpad.com>)
2. ImageJ (<https://imagej.nih.gov/ij/index.html>)

Procedure

Note: The experiment should be carried out in a biosafety level 2 Lab.

A. Preparation of bacteria

Day 1

1. Prepare or melt tryptic soy agar (TSA), as per manufacturer's instructions, and let it cool down to about 45°C (e.g., in a water bath already set at 45 °C). Add sterile Congo red solution (see Recipes) to a final concentration of 0.01%, e.g., 5 ml of Congo red solution to 500 ml of TSA. Pour the plates (about 25 ml of TSA in each 10 cm diameter plate) next to an open flame or in a sterile cabinet and let them dry. Store the plates at 4°C and use them within 3 weeks.
2. In the afternoon, streak out *Shigella flexneri* from the glycerol stock (see Notes) on a TSA plate supplemented with Congo red. Grow overnight (approximately 18 h) at 37 °C.

Day 2

3. Inoculate 5 ml of TSB with a single red colony (Figure 1) of *Shigella flexneri* and incubate overnight at 37 °C with 180 rpm agitation.

Day 3

4. Dilute the overnight culture 200 times in fresh TSB in a conical flask. For instance, in a 125 ml flask, take 25 ml of TSB and add 125 µl of the overnight culture. Incubate at 37 °C with 180 rpm agitation and grow till mid log-phase ($OD_{600nm} = 0.3-0.4$). The doubling time of *Shigella* during the exponential phase is about 40 min. It typically takes about 3 h to reach the mid log-phase.

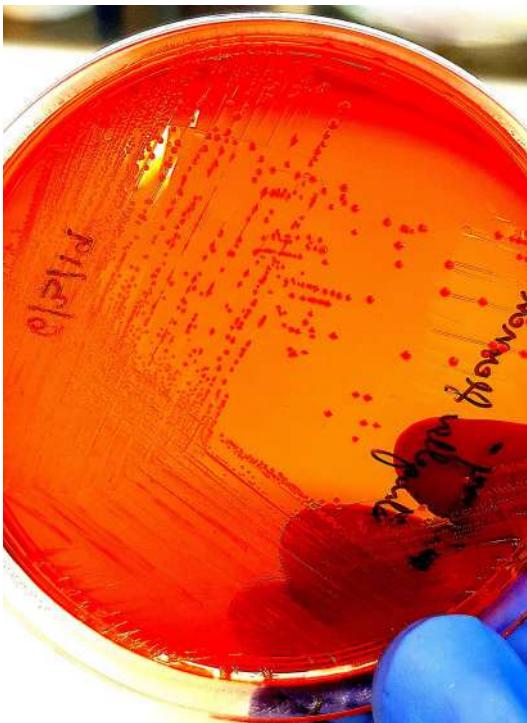


Figure 1. Characteristic red colonies of *Shigella flexneri* on TSA supplemented with 0.01% Congo red

B. Preparation of TC7 cells

Day 2

1. Remove the growth medium (see Recipes) from a cell culture flask with a confluent monolayer of TC7 cells.
2. Wash the cells with sterile PBS three times.
3. Add 5 ml of pre-warmed Trypsin solution and incubate the flask at 37 °C for about 4 min.
4. Add 5 ml of growth medium (see Recipes) to inhibit the Trypsin and collect the trypsinized cells in a 50 ml centrifuge tube.
5. Centrifuge at 900 $\times g$ and discard the supernatant. Re-suspend the pellet in 5-10 ml of growth medium devoid of antibiotics (see Recipes) by gentle pipetting.
6. In a sterile 1.5 ml microcentrifuge tube, mix 10 μl of cell suspension with 10 μl of Trypan blue solution. Load 10 μl of this mixture on to the Countess II counting slide and using a Countess II cell counter, determine the number of cells.
7. Seed the required number of wells in a 6-well plate with 1 million cells (1×10^6) per well, adding at least 2 ml of growth medium devoid of antibiotics (see Recipes) in each well. This number of cells is enough to form a fully confluent monolayer after one day of growth (Figure 2).

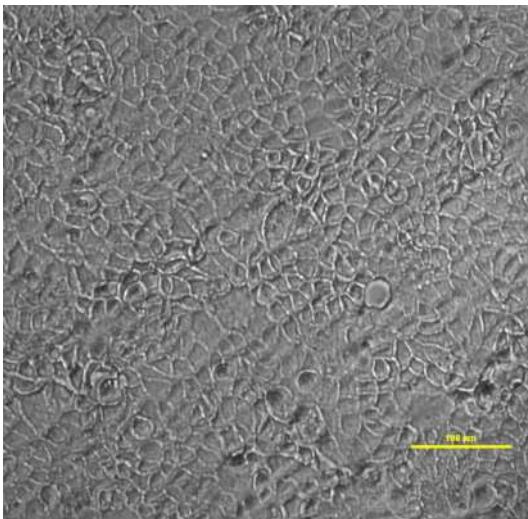


Figure 2. Bright-field image of a monolayer of TC7 cells

C. Infection

Day 3

1. On Day 3, when the bacteria reach $OD_{600nm} = 0.3\text{-}0.4$, pipette 1-1.5 ml culture into a microcentrifuge tube.
2. Spin the tube at $3,000 \times g$ for 5 min. Aspirate the supernatant. Add an equal volume of PBS at room temperature and resuspend the pellet by pipetting up and down or by gentle vortexing.
3. Repeat Step C2 at least twice and finally resuspend the pellet in infection buffer at room temperature. Record the OD_{600nm} of the bacterial suspension.

The recommended multiplicity of infection (MOI) for this experiment is very low, 1 bacterium for every 150 cells. Since there are 1×10^6 cells in each well, the number of bacteria required per well is about 6,700.

The number of *Shigella* cells at $OD_{600nm} = 1$ is $0.5 \times 10^9/\text{ml}$; hence at $OD = z$, the number of bacterial cells = $z \times 0.5 \times 10^9$. Calculate the volume of bacterial suspension required for infecting each well using the following formula:

$$\text{Volume of bacterial suspension required (in ml)} = 6,700/z \times 0.5 \times 10^9$$

Additionally, make 10-fold serial dilutions of the bacterial suspension and spread 100 μl of each on TSA plates. Incubate the plates at 37 °C and count the colonies obtained the next day. This colony count of the inoculum will verify the number of bacteria used.

4. Aspirate the medium from the TC7 cells plate and add 2 ml of PBS at room temperature to each well.
5. Swirl the plate gently and carefully aspirate all the liquid. Wash the cells twice in a similar fashion and add 2 ml of infection buffer (see Recipes) at room temperature to each well.
6. Add the desired volume of bacterial suspension (calculated in Step C3) to each well of the 6-well plate. Mix well by gentle pipetting to ensure that the bacterial suspension is mixed uniformly.

Centrifuge the plate (using the plate adaptors in the centrifuge rotor) at $180 \times g$ for 10 min at room temperature.

7. Incubate the plate at 37°C , 10% CO_2 , for 2 h.
8. After incubation, wash the wells with prewarmed PBS three times as in Steps C4 and C5.
9. Completely aspirate the liquid from the wells and cover cells with 2 ml of the cooled agarose overlay (see Recipes).
10. Put the plates back at 37°C , 10% CO_2 . Examine the plates daily for appearance of plaques. Two days incubation can be enough for optimal plaque size (1-2 mm diameter). However, it is dependent on the bacterial strain and frequent observation is therefore required.

D. Visualization and enumeration of plaques

Day 5 or 6

1. After 48-72 h of incubation, very carefully peel off the agarose overlay using a scalpel to detach the overlay from the walls and by turning around the plate.
2. Fix the cells by incubation in 95% ethanol for 5 min.
3. Remove the ethanol and stain the wells with 5% Giemsa solution (see Recipes) for 10 min.
4. Aspirate the stain and wash the wells twice with distilled water. Let the plate air-dry.
5. When the plate is dry, take a picture of the plate using an imaging system (Figure 3).
6. Count the number of plaques formed in each well with a marker pen.

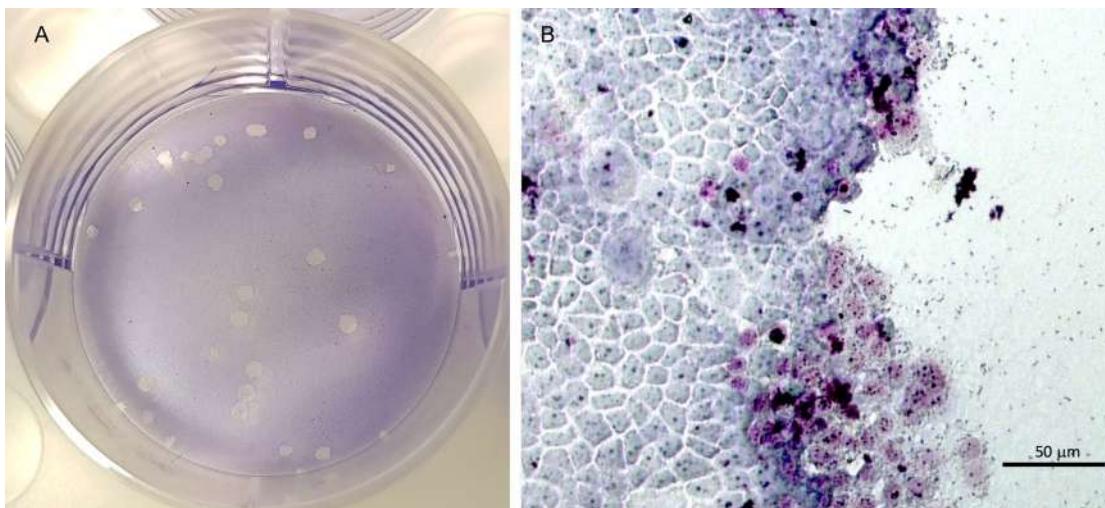


Figure 3. Visual assessment of the plaques formed on the cellular monolayer. A. Clear plaques representing dead cells in a well of 6-well plate with TC7 monolayer stained with Giemsa. B. Bright-field micrograph of a plaque edge. The clear zone is the plaque and the interface of plaque and the TC7 monolayer shows characteristic damage to the cells.

Data analysis

1. Using the ImageJ software determine the diameter of each plaque in the image.
2. Using the Prism software plot the number or the average diameter of plaques against the condition (e.g., the strain) or the MOI used in particular well (Figure 4). For every condition tested, prepare at least duplicate samples in every experiment to serve as the technical replicates. For biological replicates, repeat the experiment on another day with the same number of bacteria and cells at least 3 times.
3. To compare only two sample values at a fixed time point, use Student's *t*-test. However, to assess the statistical significance of multiple samples across multiple time points, use two-way ANOVA with Tukey's *post-hoc* test.

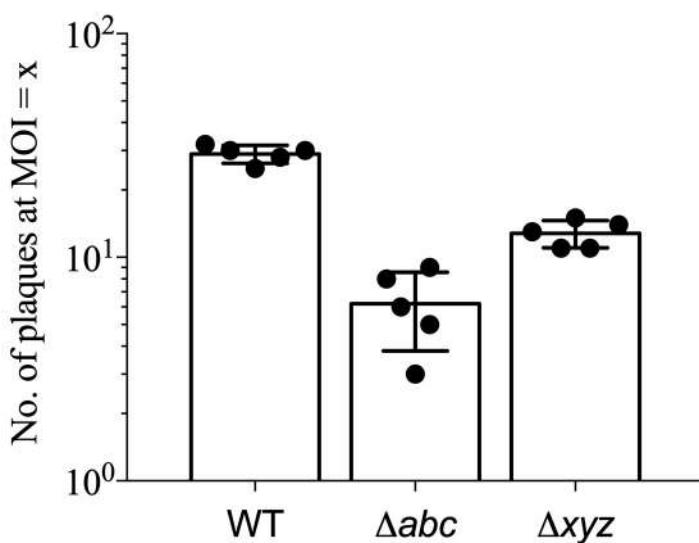


Figure 4. An example plot of the number of plaques at a particular MOI for different bacterial strains. Each dot represents an independent value obtained from a replicate.

Notes

1. To prepare glycerol stocks of *Shigella flexneri*, dilute the overnight culture 200 times in fresh TSB and incubate at 37 °C with 200 rpm shaking. Monitor the OD₆₀₀ of the culture periodically. At OD₆₀₀ = 0.6, withdraw 1 ml of culture in a 2 ml cryovial and add sterile glycerol to a concentration of 15-50% (v/v). Mix gently and store the tube at -80 °C. The frozen stocks are stable for years at -80 °C, however, multiple freeze-thaw cycles may reduce the shelf life.
2. Carefully select only the red colonies on Congo red agar for the experiment. The off-white colonies have lost the virulence plasmid and are invasion-deficient. If plates are not used immediately after overnight culture, but kept at 4 °C (which is possible for up to 3 weeks), white colonies need to be marked because they will turn red due to unspecific binding of Congo Red. Red staining of invasive colonies only develops during growth above 35 °C.

3. Congo red is able to induce secretion via the Type III Secretion System (T3SS) in *Shigella*. The dye binds to the bacterial colonies that have an active T3SS making them appear red in color. It is a simple and quick screening method to differentiate virulent *Shigella* colonies from the avirulent ones (due to curing of the virulence plasmid). Unfortunately, the mechanism of action of Congo red is not understood.
4. The TC7 cells are cultured and maintained in presence of Penicillin and Streptomycin (100 U/ml). However, infections are carried out in infection buffer that does not contain any antibiotics (which may inhibit the bacterial growth). Ideally, cells are seeded in medium devoid of antibiotics if used the next day or at least extensively washed at the beginning of the experiment.
5. The multiplicity of infection (MOI) for this experiment is to be kept very low, because neither the plaque number nor the plaque size can be determined properly when plaques are too close to each other. The MOI can be empirically determined by carrying out experiments with various dilutions of the bacterial suspension. We recommend one bacterial cell per 150 or 300 TC7 cells.
6. The 6-well plates are centrifuged after addition of bacteria to aid in binding as *Shigella* lacks adhesion factors.
7. The agarose overlay should not be hot while pouring. It may kill the cells.
8. Even during longer incubations, gentamicin won't be able to penetrate the cells and kill the intracellular bacteria. The intracellular bacteria remain 'protected' all the time.
9. Keep all the buffers and reagents that are required at the initiation of invasion and thereafter, warmed up to 37 °C before using. The secretion of T3SS effectors mediating invasion is temperature dependent. It is active above 35 °C, but inactive at room temperature. In order to synchronize the invasion, it is important to keep the bacteria in buffers at room temperature before invasion.
10. All the reagents can be prepared on Day 1, except for the agarose overlay and Giemsa solution, which must be prepared freshly.
11. When using the pipette controller, gently dispense the solutions with gravity ("G" setting in Accuripipette) or at the lowest flow speed into the 6-well plate to avoid detaching the cell monolayer.
12. Be careful while swirling the plate, washing the cells and during centrifugation, because the confluent cell monolayer detaches easily.
13. The average number and diameter of the plaque cannot be substituted with the measure of total plaque area. The number of plaques actually reflects the number of invasion sites. The diameter of the plaque reflects the capacity of the bacterium to disseminate. Therefore, total plaque area measurement won't allow distinguishing between a bacterial strain that is poorly invasive but good at disseminating from a strain that is proficient in invasion but is poor at disseminating.
14. For further reading and details on basic methods that have not been described in this manuscript (such as bacterial inoculation, pouring the plates, etc.) the reader is referred to the following books: Microbiology: A Laboratory Manual (Cappuccino and Welsh, 2017) and Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications (Freshney, 2010).

Recipes

1. Congo red solution

1% (w/v) Congo red in water

Weigh 1 g of Congo red dye and dissolve it in 70 ml of water. Bring the volume of the solution to 100 ml and filter sterilize the solution, using 0.2 µm membrane filters. The Congo red solution is stable for a long time at room temperature

2. Growth medium

DMEM

1x amino acid solution

100 U/ml Pen-Strep solution

10% heat inactivated FBS

To 500 ml DMEM, add 5 ml of Pen-Strep solution, 5 ml of amino acid solution and 50 ml of FBS (inactivated by incubation at 55 °C for 30 min)

Note: The growth medium must be stored at 4 °C and can be used for 3-4 weeks.

3. Infection buffer

DMEM (serum free)

20 mM HEPES, pH 7.4

To 50 ml of serum free DMEM add 1 ml of 1 M sterile HEPES pH 7.4

Note: Infection buffer is stable at 4 °C for months.

4. Agarose overlay

0.5% (w/v) agarose in infection buffer

5% FBS

50 µg/ml gentamicin

a. Add 0.1 g of agarose to 20 ml of infection buffer

b. Melt the agarose in a microwave oven

c. When it cools down to about 50 °C, add 1 ml of heat-inactivated FBS and 20 µl of 50 mg/ml gentamicin solution

d. Allow the agarose to cool down to about 45 °C in a water bath, so that it is still liquid but not too hot

Note: Agarose overlay needs to be prepared fresh every time before use and cannot be stored.

5. 5% (v/v) Giemsa solution

Prepare a 20 ml working Giemsa solution by using 1 ml of stock Giemsa solution to 19 ml of PBS

Keep it in a dark bottle/tube

Note: Do not store it and always use it as a freshly prepared solution.

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Competing interests

The authors declare no conflict of interest.

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QUEEN-based Spatiotemporal ATP Imaging in Budding and Fission Yeast

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[Abstract] Yeasts have provided an exceptional model for studying metabolism and bioenergetics in eukaryotic cells. Among numerous metabolites, adenosine triphosphate (ATP) is a major metabolite that is essential for all living organisms. Therefore, a clearer understanding of ATP dynamics in living yeast cells is important for deciphering cellular energy metabolism. However, none of the methods currently available to measure ATP, including biochemical analyses and ATP indicators, have been suitable for close examinations of ATP concentrations in yeast cells at the single cell level. Using the recently developed ATP biosensor QUEEN, which is suitable for yeasts and bacteria, a protocol was described herein to visualize ATP concentrations in living budding and fission yeast cells. This simple method enables the easy and reliable examination of ATP dynamics in various yeast mutants, thereby providing novel molecular insights into cellular energy metabolism.

Keywords: ATP, Budding yeast, Fission yeast, QUEEN, Biosensor, Energy metabolism, Mitochondria, Live cell imaging

[Background] Adenosine triphosphate (ATP) is one of the major and indispensable metabolites for all living organisms. In addition to being useful “energy currency” for various cellular processes, ATP serves as an intracellular and extracellular signaling molecule as well as a phosphate donor for protein phosphorylation (Lehninger *et al.*, 2010). Recent studies suggested that ATP itself solubilizes protein as a “biological hydrotrope” (Patel *et al.*, 2017) and influences the aggregation state of intracellular proteins (Pu *et al.*, 2019; Sridharan *et al.*, 2019). Due to the obvious importance of ATP and its wide involvement in many cellular events, the spatial distributions and temporal dynamics of ATP in living organisms need to be clarified in order to elucidate energy metabolism and signal transductions at the molecular level. To date, a number of methods have been developed to measure ATP concentrations *in vitro*. However, these biochemical methods have only a limited ability to examine ATP dynamics *in vivo* because of their insufficient time resolution relative to the rapid turnover of ATP (less than one minute) (Mortensen *et al.*, 2011; Takaine *et al.*, 2019). Moreover, although these biochemical analyses detect the average ATP level of a cell population, they are incapable of showing the intracellular or intratissue distribution of ATP.

To overcome these difficulties, several types of ATP indicators have been developed for the direct visualization of ATP in living organisms (Dong and Zhao, 2016). Among them, genetically encodable ATP indicators are advantageous because their introduction into a cell population is non-invasive and highly specific. A series of fluorescence resonance energy transfer (FRET)-based genetically encoded

ATP indicators, named “the ATeam”, was developed for the first time a decade ago by the pioneering work of Imamura’s group (Imamura *et al.*, 2009). The ATeam comprises the ATP-binding domain of the ε subunit of bacterial F_oF₁ ATP synthase and flanking cyan and yellow fluorescent proteins (FPs), and is now widely and successfully used to monitor intracellular ATP concentrations in various types of cells ranging from plants to mammals. However, the ATeam composed of two FPs may be susceptible to proteolysis and, thus, yield immature sensor molecules because of differences in the maturation times of the two FPs. These features manifest when the ATeam is expressed in fast-growing microorganisms, in which protein turnover is very rapid, such as yeasts or bacteria, and become problematic because the presence of a dysfunctional sensor inevitably affects the FRET signal. To circumvent the drawbacks associated with ATeam, the second-generation ATP indicator “QUEEN” (quantitative evaluator of cellular energy) was recently developed by Imamura’s group (Yaginuma *et al.*, 2014). QUEEN is a dual-excitation ratiometric ATP biosensor composed of a single FP and, thus, is more resistant to degradation and matures more rapidly than ATeam, yielding the reliable quantification of ATP, even in actively proliferating bacterial cells.

Yeasts have provided an exceptional model for studying metabolism and bioenergetics in eukaryotic cells. Yeast carbon metabolism has long been studied because of its industrial importance for producing many valuable chemicals (yeast metabolites) (Borodina and Nielsen, 2014). Energy metabolism in yeasts also provides a tractable model for studying energy metabolism in tumor cells because they are similar in that they synthesize the majority of ATP through glycolysis and not oxidative phosphorylation, even in the presence of oxygen, and this is known as “aerobic glycolysis” or “the Warburg effect” (Diaz-Ruiz *et al.*, 2011). Therefore, a close examination of ATP dynamics in living yeast cells has been of great significance, but waits to be embarked due to the absence of an appropriate ATP biosensor.

In a recent study, QUEEN was applied to both the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* for the first time and intracellular ATP levels and its dynamicity were visualized in wild-type cells (Takaine *et al.*, 2019). The use of QUEEN enables the easy and reliable examination of ATP dynamics in living yeast cells, providing novel insights into cellular energy metabolism. The protocols used to observe and analyze the QUEEN fluorescence signal in yeast cells have been described herein. The constitutive and strong expression of QUEEN did not induce any noticeable defects in cell growth, meiosis, or sporulation. Thus, ATP concentrations in various yeast mutants under a number of conditions may be observed, as well as ATP in wild-type cells, using the same method. Moreover, this method employs a conventional wide-field fluorescence microscope and free image processing software for image calculations, and, thus, is accessible to a broad range of researchers.

Materials and Reagents

1. 35-mm glass-bottomed dish (IWAKI, catalog number: 3971-035, No. 1.5 thickness)
2. Cell culture dish, sterilized (SANSEI MEDICAL CO., LTD., catalog number: 01-013)
3. Pipette tips (Watson® Bio Lab, catalog numbers: 123R-755YS [200 µl], 123R-757CS [1,000 µl])
4. Sterile culture tube (16 ml, polypropylene) (EVERGREEN, catalog number: 222-2393-080)
5. Toothpicks (6.5-18 cm) (any brand)
Note: Sterilize before use. Reusable until they break.
6. Budding yeast cells expressing QUEEN-2m:
e.g., MTY3261 (MAT α his3Δ1::3x $pRS303\text{-}P_{TEF1}\text{-}QUEEN\text{-}2m\text{-}T_{CYC1}$ leu2Δ0 ura3Δ0) from Takaine et al., 2019. This strain was derived from a slightly modified BY4741 parental strain (MAT α his3Δ1 leu2Δ0 ura3Δ0).
7. Fission yeast cells expressing QUEEN-2m:
e.g., KSP3769 (h^r leu1-32:: $P_{tif51}\text{-}QUEEN\text{-}2m\text{:}leu1^+$) from Ito et al., 2019.
Note: Both yeast strains expressing QUEEN-2m and plasmids for expressing QUEEN-2m in budding yeast are available from the Yeast Genetic Resource Centre Japan (YGRC, <http://yeast.nig.ac.jp/yeast/>).
8. Concanavalin A (Sigma-Aldrich, catalog number: C-7275)
9. Soybean lectin (Sigma-Aldrich, catalog number: L-1395)
10. Yeast nitrogen base w/o amino acids (YNB) (Invitrogen, catalog number: Q300-09)
11. Yeast extract (BD, catalog number: 212750)
12. Bacto-peptone (BD, catalog number: 211677)
13. D(+)-Glucose (FUJIFILM Wako, catalog number: 049-31165)
14. 2-Deoxy-D-glucose (2DG) (FUJIFILM Wako, catalog number: 046-06483)
Note: 2DG is a potent inhibitor of glycolysis and has been used to deplete ATP in yeast cells (Serrano, 1977; Xu and Bretscher, 2014; Takaine et al., 2019).
15. Agar (FUJIFILM Wako, catalog number: 010-08725)
16. Potassium hydrogen phthalate (FUJIFILM Wako, catalog number: 163-03822)
17. Na₂HPO₄·12H₂O (Kishida Chemical Co., Ltd., catalog number: 000-72535)
18. Ethanol (Kishida Chemical Co., Ltd., catalog number: 140-28553)
19. NH₄Cl (FUJIFILM Wako, catalog number: 017-02995)
20. Uracil (FUJIFILM Wako, catalog number: 212-00062)
21. Myo-inositol (FUJIFILM Wako, catalog number: 096-00285)
22. L-Glutamic acid (FUJIFILM Wako, catalog number: 072-00501)
23. Adenine sulfate (FUJIFILM Wako, catalog number: 018-19613)
24. L-Leucine (PEPTIDE INSTITUTE INC., catalog number: 2713)
25. L-Histidine (FUJIFILM Wako, catalog number: 082-00683)
26. L-Lysine·HCl (PEPTIDE INSTITUTE INC., catalog number: 2714)
27. L-Methionine (PEPTIDE INSTITUTE INC., catalog number: 2715)
28. L-Phenylalanine (PEPTIDE INSTITUTE INC., catalog number: 2717)
29. L-Serine (PEPTIDE INSTITUTE INC., catalog number: 2719)

30. L-Threonine (PEPTIDE INSTITUTE INC., catalog number: 2720)
31. MgCl₂·6H₂O (FUJIFILM Wako, catalog number: 132-00175)
32. CaCl₂·2H₂O (FUJIFILM Wako, catalog number: 033-25035)
33. KCl (FUJIFILM Wako, catalog number: 163-03545)
34. Na₂SO₄ (FUJIFILM Wako, catalog number: 195-03341)
35. H₃BO₃ (FUJIFILM Wako, catalog number: 021-02195)
36. MnSO₄·5H₂O (FUJIFILM Wako, catalog number: 139-00825)
37. ZnSO₄·7H₂O (FUJIFILM Wako, catalog number: 268-00405)
38. FeCl₃·6H₂O (FUJIFILM Wako, catalog number: 095-00875)
39. Na₂MoO₄·2H₂O (FUJIFILM Wako, catalog number: 198-02471)
40. KI (FUJIFILM Wako, catalog number: 166-03971)
41. CuSO₄·5H₂O (FUJIFILM Wako, catalog number: 031-04411)
42. Citric acid (FUJIFILM Wako, catalog number: 038-05521)
43. Nicotinic acid (FUJIFILM Wako, catalog number: 142-01232)
44. Biotin (TOKYO CHEMICAL INDUSTRY CO., LTD., catalog number: B0463)
45. Sodium pantothenate (FUJIFILM Wako, catalog number: 198-05651)
46. 100x Amino acids mix (see Recipes)
47. 100x L-Leucine stock (see Recipes)
48. 50x Uracil stock (see Recipes)
49. SC-His liquid medium (see Recipes)
50. YPD agar plate (see Recipes)
51. YELA agar plate (see Recipes)
52. 100x 5 Low-supplements (see Recipes)
53. 50x Salt stock (see Recipes)
54. 1,000x Vitamins (see Recipes)
55. 10,000x Minerals (see Recipes)
56. EMM liquid medium (see Recipes)
57. 2-Deoxy-D-glucose (2DG) medium (see Recipes)
58. Glass-bottomed dish coated with concanavalin A (see Recipes)
59. Glass-bottomed dish coated with soybean lectin (see Recipes)

Equipment

1. Inverted fluorescent microscope (Nikon, Eclipse Ti-E) equipped with:
 - a. A 100x objective lens (Nikon, Apo TIRF 100x Oil DIC N2/NA 1.49)
 - b. A high-pressure mercury lamp (Nikon, Intensilight C-HGFIE 130W)
 - c. An electron multiplying charge-coupled device camera (Andor-Oxford Instruments, iXon3 DU897E-CS0-#BV80)
 - d. A FITC filter set (Nikon, Ex465-495/DM505/BA515-555)

- e. A custom-made Ex409 filter set (Semrock, Ex393-425/DM506/BA516-556)
- f. Neutral density (ND) filters (Nikon, ND4 [MBN21804] and ND8 [MBN21808])
2. Bench-top aspirator (INTEGRA, VACUSIP)
3. Inverted routine microscope (Nikon, Eclipse Ts2) equipped with a 40× objective lens (Nikon, CFI Achromat LWD ADL 40XF/NA 0.55, catalog number: MRP46402)
4. Autoclave (TOMY SEIKO CO., LTD, LBS-325)
5. Pure water system (Merck Millipore, Milli-Q® Integral 15)
6. Erlenmeyer flasks (50 ml) (HARIO, catalog number: 82-0144)
7. Silicone stopper (for a 50-ml flask) (Shin-Etsu Polymer, SILICOSEN T22)
8. Incubator (TAITEC, INCUBATE BOX M-200F)
9. Culture rotator (TAITEC, RT-50)
10. Rotary shaker (NISSIN, NX-20)
11. Pipettes (BM EQUIPMENT Co., LTD, PipetPAL®, model: PAL-200 [20-200 µl], PAL-1000 [100-1,000 µl])

Software

1. NIS-Element AR ver. 4.30.01 (Nikon, https://www.microscope.healthcare.nikon.com/ja_JP/)
2. Fiji ver. 2.0.0-rc-69/1.52n (Fiji contributors [Schindelin *et al.*, 2012], <http://fiji.sc/#>)
3. Microsoft® Excel for Mac 2011 ver. 14.7.3 (Microsoft, <https://products.office.com/ja-jp/excel>)
4. KaleidaGraph ver. 4.5.1 (HULINKS, https://www.hulinks.co.jp/software/stat_graph/kaleida)

Procedure

- A. Cell preparation
 1. Streak yeast cells from a frozen stock using a pipette tip on a YPD (for *S. cerevisiae*) or YELA (for *S. pombe*) agar plate, and incubate at 30 °C for 1-2 days.
 2. Preparation of the preculture:
 - a. For budding yeast, inoculate 2 ml of SC-H with a loopful of cells in a 16-ml culture tube using a sterilized toothpick, and culture at 30 °C overnight with rotation at approximately 40 rpm using a rotator.
 - b. For fission yeast, inoculate 2 ml of EMM with a loopful of cells in a 16-ml culture tube using a sterilized toothpick, and culture at 30 °C overnight with shaking at 150 rpm using a rotary shaker.
 3. Preparation of the main culture:
 - a. For budding yeast, dilute 0.15 ml of the overnight culture with 2.85 ml SC-H (1:20) in a 16-ml culture tube, and culture at 30 °C with rotation at approximately 40 rpm.
 - b. For fission yeast, dilute 0.5 ml of the overnight culture with 9.5 ml EMM in a 50-ml flask (1:20), and culture at 30 °C with shaking at 150 rpm.

4. Grow yeast cells to the mid-log phase. Approximately 3 h is needed for budding yeast and approximately 5 h for fission yeast under the experimental conditions described herein. The dilution ratio and incubation time may be optimized according to individual culture systems.
5. Dilute the cell culture with the same medium by 10-fold and place 100 μ l of cells onto a 35-mm glass-bottomed dish coated by concanavalin A for *S. cerevisiae* or soybean lectin for *S. pombe* (see Recipes).
6. Wait for 5 min, remove most of the medium with gentle aspiration, and wash cells immobilized on the glass surface with 300 μ l of fresh medium three times.
7. Observe the yeast cells immobilized on the glass-bottomed dish with a 40x objective lens using an inverted routine microscope to check cell density.
8. Repeat Steps A2-A3 if cells are too sparse.
9. Fill the dish with 4-5 ml of medium and incubate the dish in a room for fluorescent microscopy (maintained at 25 °C) for at least 30 min before microscopic observations.
10. Optional: To deplete intracellular ATP, replace the medium with 300 μ l of 2DG medium three times. Acquire images of QUEEN fluorescence within 10 min.

Note: The dish is filled with an excess amount of medium relative to the cell volume in order to minimize changes in the chemical composition of the medium by the consumption of nutrients or by secretory components during observations. It is important to maintain the medium in a dish at a constant and stable temperature because the affinity of QUEEN for ATP depends on temperature (Yaginuma et al., 2014): higher temperatures result in lower QUEEN ratios, while lower temperatures lead to higher QUEEN ratios.

B. Image acquisition

1. Power on the fluorescent microscope system at least 1 h before imaging to reach a stable and constant temperature of approximately 25 °C.
2. Observe the yeast cells immobilized on the glass-bottomed dish with a 100x objective lens using an inverted fluorescence microscope by bright-field imaging and focus the cells.
3. Take a bright-field image and two fluorescence images of QUEEN using FITC (ex480 image) and Ex409 (ex409 image) filter sets from a single z-plane.
4. Regarding time-lapse imaging, reduce the intensity of the excitation light and exposure times (see Table 1 for details) to prevent the photobleaching of QUEEN fluorescence and minimize damage to cells.
5. Save the combined images (bright-field and QUEEN) as an nd2 file.

Note: The bright-field image is dispensable for calculating the QUEEN ratio, but is useful for examinations of cell viability or cell boundaries. Red fluorescence (emission wavelength > 620 nm) is compatible with QUEEN fluorescence. In single time-point imaging, collect images of cells from several fields of view for each experimental condition, giving a sufficient sample size for a quantitative analysis. Observations of 3-5 fields yields n > 100 cells in total are appropriate. The parameters used for the fluorescence microscopy of QUEEN are summarized in Table 1.

Table 1. Parameters for the fluorescence microscopy of QUEEN

	Ex480	Ex409
Binning	1 × 1 (no binning)	
Exposure time (ms)*		
single time-point	900/1800	150/300
time-lapse	300/900	50/150
Readout mode	EM gain 3 MHz 14-bit	
EM gain Amplifier	200	
Conversion gain	5×	
ND filter for fluorescence excitation light		
single time-point	ND32 (Eclipse Ti-E) × ND16 (C-HGFIE)	
time-lapse	ND32 (Eclipse Ti-E) × ND32 (C-HGFIE)	

*Exposure time for the acquisition of images of QUEEN expressed in budding/fission yeast.

Data analysis

In the following sections, menu commands and options in Fiji software are expressed in **bold** font.

A. Calculation of the QUEEN ratio (single time-point imaging)

1. Open an nd2 file using Fiji software as a hyperstack file (multi-channels and one time frame) and split the hyperstack into each channel image (one bright-field image, two QUEEN images): **Image -> Color -> Split Channels** (Figure 1A, raw image).
2. Subtract the background (pixel values in an area outside the cells) of QUEEN images using the rolling-ball algorithm (Figure 1A, background-subtracted): **Process -> Subtract Background**. Set the **rolling ball radius** 20 (for *S. cerevisiae*) or 50 (for *S. pombe*) pixels and keep all the check boxes unchecked.
3. Convert QUEEN images to a signed 32-bit floating-point grayscale: **Image -> Type -> 32-bit**
4. Assess the maximum and minimum thresholds of the images using the modified IsoData algorithm (default algorithm): **Image -> Adjust -> Threshold**. Check the **Dark background** check box and click **Auto** and **Apply**. Check **Set background pixels to NaN** to set background (non-thresholded) pixels to the *NaN* (Not a Number) value (Figure 1A, thresholded).
5. Divide the pixel values of the ex409 image by those of the ex480 image to calculate the QUEEN ratio at each pixel: **Process -> Image calculator, Image1: ex409 image, Operation: Divide, Image2: ex480 image**.
6. Apply the rainbow smooth look-up table (or any other table if preferable) to the ratio image to express the QUEEN ratio in pseudocolor: **Image -> Lookup Tables -> Rainbow Smooth**.
7. Adjust the display range to include the minimum and the maximum values of the QUEEN ratios in the image: **Image -> Adjust -> Brightness/Contrast -> Set**. Set **Minimum displayed value** 0.5 and **Maximum displayed value** 3, respectively, for example (Figure 1B, ratio image).

8. Save the ratio image and the bright-field image as a tiff file: **File -> Save as...**
9. Optional: steps 1-7 may be automated by running the following macro: **Plugins -> Macros -> Install...**, select and open stack2ratio.jim macro. Open an nd2 file and apply **Run** on the macro window.

QUEEN ratio calculation macro (stack2ratio.jim)

```
// Macro starts here  
  
title = getTitle();  
run("Split Channels");  
c1 = "C1-" + title;           // channel #1: bright-field image  
c2 = "C2-" + title;           // channel #2: ex480 image  
c3 = "C3-" + title;           // channel #3: ex409 image  
  
selectWindow(c2);  
run("Subtract Background...", "rolling=20 stack");  
run("32-bit");  
setAutoThreshold("Default dark");  
run("NaN Background", "stack");  
  
selectWindow(c3);  
run("Subtract Background...", "rolling=20 stack");  
run("32-bit");  
setAutoThreshold("Default dark");  
run("NaN Background", "stack");  
  
imageCalculator("Divide create stack",c3,c2);  
run("Rainbow Smooth");  
setMinAndMax(0.5, 3); //  
rename("Ratio-" + title);  
// Macro ends here
```

Note: The optimal rolling ball radius depends on the spatial resolution of the imaging system (0.16 μm/pixel in our system) and the average size of yeast cells examined. Therefore, readers need to adjust the rolling ball radius according to their own imaging system by trial and error.

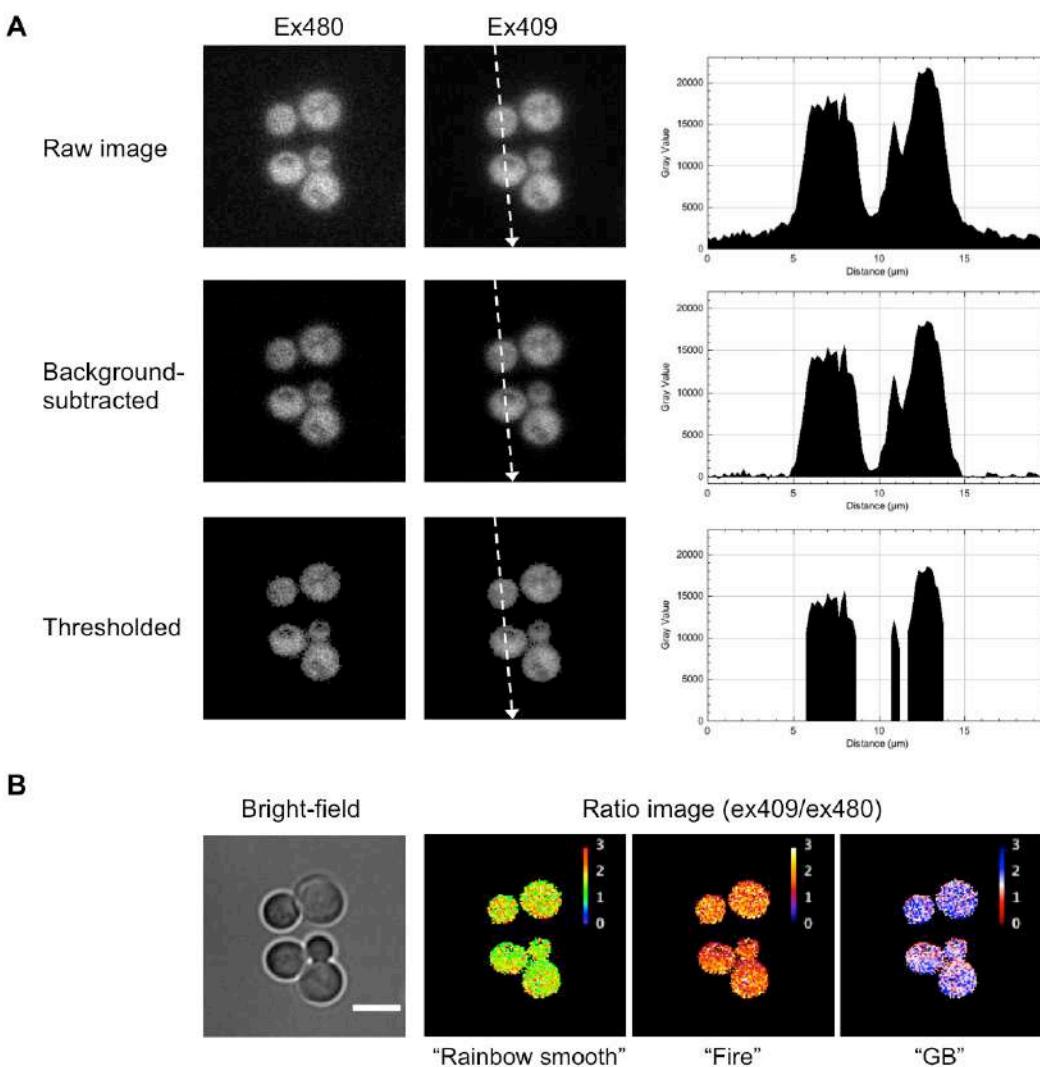


Figure 1. Generation of a QUEEN ratio image. A. Image processing of images of budding yeast cells expressing QUEEN. Right panels show the line profile of pixel values along dotted lines indicated in the ex409 images. B. The bright-field image and QUEEN ratio images calculated from the ex480 and ex409 images shown in (A) expressed in three different lookup tables. White scale bar = 5 μ m.

B. Calculation of the QUEEN ratio (time-lapse imaging)

1. Open the nd2 file using Fiji software as a hyperstack file (multi-channels and multiple time frames).
2. By choosing a **Rectangle** selection tool (see Figure 2A), draw the region of interest (ROI) to include a cell (and the progeny cells if necessary) throughout the time course.
3. Create a new hyperstack file composed of the ROI: **Image -> Duplicate**. Check the **Duplicate hyperstack** check box and preserve all **Channels (c)** and **Frames (t)**.
4. Split the hyperstack into each channel stack (containing time frames): **Image -> Color -> Split Channels**.

5. Subtract the background using the rolling-ball algorithm as described in step A2. Process all the images in the stack.
6. Convert the QUEEN stacks to a signed 32-bit floating-point grayscale: **Image -> Type -> 32-bit**
7. Display the last time frame: **Image -> Stacks -> Set Slice**. Enter the last slice number.
Note: Against a stack of 32-bit images, the maximum and minimum thresholds are calculated using the currently displayed image (slice), and not calculated for each image. Assessments of thresholds using the last time frame were appropriate under the experimental conditions described herein.
8. Create thresholded stacks as described in step A4.
9. Calculate and save the QUEEN ratio stack as described in steps A5-A8.

C. Measurement of the mean QUEEN ratio in individual cells (single time-point imaging)

1. Open the QUEEN ratio image using Fiji software (Figure 2B).
2. By choosing the **Rectangle** or **Polygon** selection tool (Figure 2A), draw the ROI to include a living cell and register the ROI in the ROI manager: **Analyze -> Tools -> ROI manager**. Click **Add** or press **t** to register the ROI (Figure 2C).
3. Repeat step 2.
4. Set the measurement: **Analyze -> Set Measurements...**, check the **Mean Gray Value** check box.
5. Select all ROI stored in the ROI manager and measure the mean of pixel values (QUEEN ratios) in ROIs: **ROI manager -> Deselect -> Measure**
6. Save the data table as a text file in the comma-separated value (csv) format: Activate the **Results** window (Figure 2D), then **File -> Save as...**

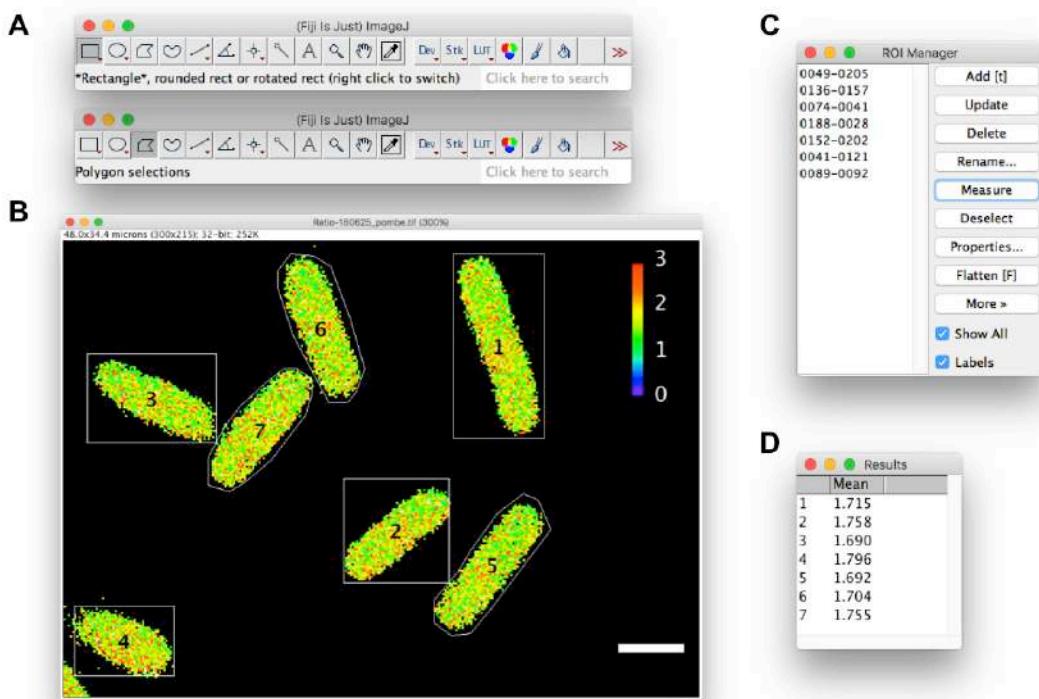


Figure 2. Measurement of the mean QUEEN ratio in yeast cells. Measurements in fission yeast cells are shown as an example. A. **Rectangle** or **Polygon** selection tool. B. A QUEEN ratio image of fission yeast cells. Outlines of cells are drawn using the rectangle (cell #1-4) or polygon (cell #5-7) selection tool. C. **ROI manager**. D. **Results** window. White scale bar = 5 μ m.

Note: Since background pixels are assigned to NaN values and, thus, ignored in the calculation of the mean of pixel values, a precise drawing of the cell outline is not necessarily required. The mean QUEEN ratio in pixels corresponding to the inside of a cell represents the cytoplasmic ATP level of the cell.

D. Measurement of the mean QUEEN ratio in a cell (time-lapse imaging)

1. Open the QUEEN ratio stack using Fiji software.
2. By choosing the **Rectangle** or **Polygon** selection tool, draw the ROI to include a cell and register the ROI in the ROI manager.
3. Repeat step 2 if necessary.
4. Select the ROI stored in the ROI manager and measure the mean of pixel values in the ROI throughout time series: **ROI manager -> More -> Multi Measure**. Check the **Measure all the slices** and **One row per slice** check boxes.
5. Save the result as described in step C6.

E. Data analysis

1. Import data into Microsoft® Excel, and calculate averages and standard deviations.
2. Calculate the *P*-value using an unpaired one-tailed Welch's *t*-test in Excel software, and

evaluate the significance of differences between two sets of data.

3. Plot data as a dot plot using KaleidaGraph software. An example of a dot plot is shown in Figure 3.

Note: The presentation of data as a dot plot appears to be the most effective way to convey distribution characteristics in extensive detail. Data generally follow a normal distribution even though there are some outliers.

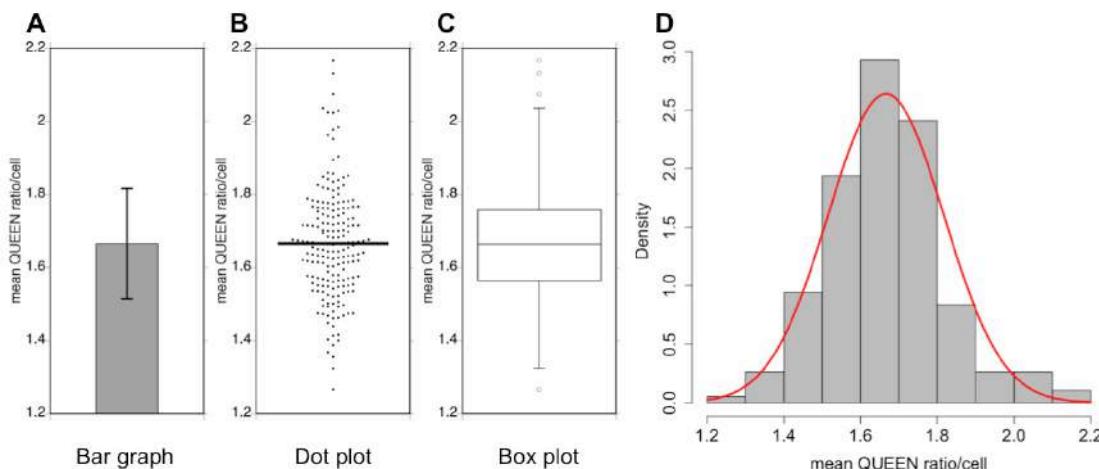


Figure 3. Examples of data analyses. A data set of measurements of the mean QUEEN ratio in budding yeast cells is represented in four different types of graphs. Sample size = 191 cells, the mean value = 1.67, variance = 0.0228, and the standard deviation (SD) = 0.151. A. Bar graph. Data are shown as the mean of the population $\pm 1 \times$ SD (error bar). B. Dot plot. Each dot corresponds to the mean QUEEN ratio inside a cell. The horizontal bar indicates the mean of the population. C. Box plot. The plot shows the 75th and 25th percentiles of the data (interquartile range) as the upper and lower edges of the box. A horizontal bar in the box indicates the median of the population. Whiskers and circles indicate the 1.5 \times interquartile range and outliers, respectively. D. Histogram. Data are shown as probability density. A normal distribution with the same mean and variance to the data is shown as a red curve.

Notes

1. The mean QUEEN ratio in a cell is calculated based on (1) the intracellular ATP concentration, (2) the affinity of QUEEN for ATP, and (3) the settings and conditions of the imaging system. Thus, many factors affect the QUEEN ratio including the conditions of the cell culture, the batch of the culture medium, temperature (as described in Procedure A), the power output of the fluorescence excitation lamp, and the conditions of optical filters (excitation, emission, and neutral density) for fluorescence microscopy. These factors vary slightly day to day and are difficult to strictly control. Therefore, comparisons of data obtained from experiments performed on the same day and not pooling data from experiments performed on different days are

recommended.

2. The readers need to optimize the imaging parameters of fluorescence microscopy for their own imaging systems. Since an excitation light of approximately 409 nm is cytotoxic and may affect the fluorescence emission spectrum of QUEEN, its intensity and exposure time need to be minimized. Adjustments to imaging parameters in order for the mean QUEEN ratio in cells depleted of ATP by 2DG, corresponding to the minimum mean QUEEN ratio, to be calculated as 0.7-0.8 are recommended. Under preferable conditions, the dynamic range of QUEEN-2m (the ratio between the maximum and minimum mean QUEEN ratios) is expected to reach 3.0-fold (Yaginuma *et al.*, 2014; Takaine *et al.*, 2019).
3. In addition to the calculation of the QUEEN ratio, measurements of the mean QUEEN ratio (the steps C1-C5) may also be automated by running a macro. Moreover, steps A1-A8 and C1-C5 in the Data analysis may be batch-processed: multiple nd2 files are automatically and sequentially processed by running a batch macro. However, the automatic drawing of cell outlines and batch processing are both beyond the scope of this study and, thus, were omitted. These macros are available from the author upon request.

Recipes

1. 100x Amino acids mix
 - a. Mix L-glutamic acid 10 g, L-lysine 12 g, L-methionine 4 g, L-phenylalanine 5 g, L-serine 38 g, L-threonine 20 g, and Myo-inositol 0.6 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
2. 100x Leucine stock
 - a. Dissolve L-leucine 11 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
3. 50x Uracil stock
 - a. Dissolve uracil 2 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
4. SC-His (according to Hanscho *et al.*, 2012)
 - a. Mix YNB 3.4 g, glucose 20 g, 100x amino acid mix 10 ml, 100x leucine stock 10 ml, and 50x uracil stock 20 ml in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
5. YPD agar plate
 - a. Mix yeast extract 10 g, glucose 20 g, Bacto-peptone 20 g, and agar 20 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min
 - c. Pour the medium into a cell culture dish (approximately 20 ml/dish)
 - d. Solidify the plates for 2-3 days before use, wrap in plastic wrap, and store at room temperature

6. YELA agar plate
 - a. Mix yeast extract 10 g, glucose 20 g, adenine sulfate 0.04 g, and agar 20 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min
 - c. Pour the medium into a cell culture dish (approximately 20 ml/dish)
 - d. Solidify the plates for 2-3 days before use, wrap in plastic wrap, and store at room temperature
7. 100x 5 Low-supplements
 - a. Mix adenine sulfate 10 g, L-lysine 3 g, L-leucine 6 g, L-histidine 2 g, and uracil 2 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
8. 50x Salt stock
 - a. Mix MgCl₂·6H₂O 53.3 g, CaCl₂·2H₂O 0.735 g, KCl 50 g, and Na₂SO₄ 2 g in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and store at room temperature
9. 1,000x Vitamins
Mix nicotinic acid 0.1 g, myo-inositol 0.1 g, biotin 0.1 mg, and sodium pantothenate 10 mg in 100 ml of absolute ethanol and store at -20 °C
Note: These components do not completely dissolve.
10. 10,000x minerals
 - a. Dissolve H₃BO₃ 0.05 g, MnSO₄·5H₂O 0.057 g, ZnSO₄·7H₂O 0.04 g, FeCl₃·6H₂O 0.02 g, Na₂MoO₄·2H₂O 0.016 g, KI 0.01 g, CuSO₄·5H₂O 0.004 g, and citric acid 0.1 g in 100 ml of deionized water
 - b. Sterilize by filtration and store at room temperature
11. EMM
 - a. Mix potassium hydrogen phthalate 3 g, Na₂HPO₄·12H₂O 5.54 g, glucose 20 g, 50x salt stock 20 ml, and NH₄Cl 5 g, and 10,000x minerals 0.1 ml in 1,000 ml of deionized water
 - b. Autoclave at 120 °C for 20 min and cool down
 - c. Add 1,000x vitamins 1 ml and store at 4 °C
 - d. Aliquot and prewarm before use
12. 2DG medium
The same as for SC-His or EMM except for the addition of 3.28 g of 2DG (final 20 mM) instead of glucose
13. Glass-bottomed dish coated with concanavalin A
 - a. Dissolve concanavalin A with sterilized deionized water at 2 mg/ml and aliquot. The solution may be stored at -20 °C
 - b. Place 100 µl of 2 mg/ml of concanavalin A solution on the glass surface of a glass-bottomed dish, and keep at room temperature for 5 min
 - c. Aspirate concanavalin A and wash the glass surface with 100 µl of sterilized deionized water

three times

- d. Dry the glass surface for 10 min before use. The dish may be stored at 4 °C
14. Glass-bottomed dish coated with soybean lectin
- a. Dissolve soybean lectin with sterilized deionized water at 2 mg/ml and aliquot. The solution may be stored at 4 °C
 - b. Place 5 µl of 2 mg/ml lectin solution on the glass surface of a glass-bottomed dish, and spread the solution using the pipette tip
 - c. Dry the glass surface for 10 min before use. The dish may be stored at 4 °C

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Competing interests

The author declares no competing interests.

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Yeast Single-cell RNA-seq, Cell by Cell and Step by Step

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[Abstract] Single-cell RNA-seq (scRNA-seq) has become an established method for uncovering the intrinsic complexity within populations. Even within seemingly homogenous populations of isogenic yeast cells, there is a high degree of heterogeneity that originates from a compact and pervasively transcribed genome. Research with microorganisms such as yeast represents a major challenge for single-cell transcriptomics, due to their small size, rigid cell wall, and low RNA content per cell. Because of these technical challenges, yeast-specific scRNA-seq methodologies have recently started to appear, each one of them relying on different cell-isolation and library-preparation methods. Consequently, each approach harbors unique strengths and weaknesses that need to be considered. We have recently developed a yeast single-cell RNA-seq protocol (yscRNA-seq), which is inexpensive, high-throughput and easy-to-implement, tailored to the unique needs of yeast. yscRNA-seq provides a unique platform that combines single-cell phenotyping via index sorting with the incorporation of unique molecule identifiers on transcripts that allows to digitally count the number of molecules in a strand- and isoform-specific manner. Here, we provide a detailed, step-by-step description of the experimental and computational steps of yscRNA-seq protocol. This protocol will ease the implementation of yscRNA-seq in other laboratories and provide guidelines for the development of novel technologies.

Keywords: Yeast, Single-cell RNA-seq, Transcriptomics, Transcript isoforms, Noncoding RNA

[Background] The appearance of single-cell-omics has revolutionized our understanding of many biological processes and is a field that is rapidly evolving at both the experimental and computational level. Over the last decade, there has been a rapid increase in the number of features that can be measured within individual cells. Single-cell RNA-seq (scRNA-seq) has pioneered this endeavor, and it has become a routine experiment to perform in higher eukaryotes. While very close to the entirety of the

repertoire of bulk experiments in higher eukaryotes now have single-cell counterparts, single-cell tools for microorganisms (beyond fluorescent reporter-based methods) are scarce. This technological gap is mainly due to technical limitations imposed by the intrinsic nature of yeast and other microorganisms.

Unlike the mammalian cells, in which scRNA-seq was developed, yeast cells are smaller in cell, genome and transcriptome size. The presence of a rigid cell wall, which needs to be removed prior to library preparation, has been one of the major technical challenges for single cell analysis. On top of that, the yeast genome is highly condensed and pervasively transcribed. Over 85% of their genome is expressed from both strands with extensive transcript isoform diversity per gene (David *et al.*, 2006; Pelechano *et al.*, 2013, 2014 and 2015), and this requires a sensitive, quantitative and strand-specific approach.

As was the case for higher eukaryotes, initial studies exploring scRNA-seq in yeast focused on the development and optimization of the method. Recently, three yeast single-cell RNA-seq approaches have been reported (Gasch *et al.*, 2017; Nadal-Ribelles *et al.*, 2019; Saint *et al.*, 2019). Interestingly, each one of them uses a different cell-isolation strategy and library-preparation protocol. Cell isolation is one of the main differences across these studies that range from microfluidics (Gasch *et al.*, 2017), micromanipulation (Saint *et al.*, 2019) and index sorting (Nadal-Ribelles *et al.*, 2019), the later described here. Microfluidic and micromanipulation-based approaches require specialized equipment and are labor intensive, but allow imaging of individual cells by microscopy as a way to measure phenotype. The protocol described here for yeast single-cell RNA-seq (yscRNA-seq), relies on index sorting as a strategy for recording phenotypic information such as cell morphology and any Fluorescent-Activated Cell-Sorting-compatible feature (fluorescent proteins, antibodies, etc.). Index sorting provides a seamless strategy for merging techniques for individual phenotyping and transcriptional profiling in cells.

A major tipping point for single-cell isolation was the appearance of droplet-based methodologies (Zhang *et al.*, 2019). These technologies dramatically increase potential throughput by generating and isolating cDNA in barcoded gel emulsions, which encapsulate single cells on the order of thousands of cells per run, and thus the cost significantly decreases. Interestingly, a preliminary droplet-based study in yeast is underway (Gresham *et al.*, 2019). While cell capturing boosts cell numbers to an unprecedented scale, the number of genes detected per cell tends to be much lower (Skinnider *et al.*, 2019) and phenotypic information of individual cells is lost. In addition, customization of library preparation is complex and labor intensive in droplet based-approaches, which will most likely be bypassed as technologies are developed.

While cell-isolation strategies determine the number of cells and phenotypic information that can be recorded, the choice of library-preparation approach defines the kind of transcriptional profiling. Due to the intertwined nature of the yeast transcriptome, and the diversity of isoforms per gene, library preparation requires both gene- and strand-isoform-specific resolutions. YscRNA-seq is based on STRT-seq (Islam *et al.*, 2014), and its library design bypasses the technical limitations of working with yeast, fulfilling the requirements for a high-resolution transcriptome profiling method. First, unique molecular identifiers (UMI) are incorporated during cDNA synthesis via a biotinylated template-switching oligo (TSO) at the 5'-end of the molecule to digitally count the absolute abundance of a gene, along with

its transcription start site (TSS) position. Second, the use of a homemade Tn5 enzyme to incorporate cell-specific adaptors provides a cost-effective strategy that greatly reduces experimental costs, compared to commercial Tn5 (Hennig *et al.*, 2018). Finally, biotinylated primers allow selectively recovering 5' ends. Moreover, the high affinity between streptavidin and biotin leads to a sequestration of the biotinylated strand after a brief denaturing step, which releases the non-biotinylated strand into the supernatant for sequencing. These features make yscRNA-seq one of the most sensitive methods available, and the most sensitive method for yeast reported to date (Nadal-Ribelles *et al.*, 2019).

We anticipate that the development of novel methodologies with different aims will soon start to emerge, and yscRNA-seq will be extended to any yeast species or microorganism. The protocol described here, along with future optimizations, could serve as a starting point for the development of new methods. There are many unanswered biological questions that influence our understanding of human health, such as the emergence of drug-resistant microbial phenotypes. Generation of solid frameworks to profile microorganisms at single-cell resolution promises to expand our understanding, and perhaps answer some of these questions once and for all.

Materials and Reagents

Note: Reagents can be from different suppliers as far as they are only used for single-cell RNA-seq protocols and nuclease free. The ones listed here were used to develop the protocol. The regular molecular biology reagents are assumed to be already present in each laboratory (e.g., water, Tris, or NaCl). For plastic labware (filter tips and tubes), we have used several brands, with identical results as far as the material is certified to be nuclease-free and low binding.

1. Eppendorf LoBind 1.5 ml (Eppendorf, catalog number: 30108051)
2. Break-away plates (Thomas Scientific, catalog number: EK-75118)
3. 96-well plates (Thomas Scientific, catalog number: EK-75012)
4. Filter Tips (Mettler Toledo, catalog numbers: 17007954, 17014973, 17014973)
5. qPCR plates (Applied Biosystems, catalog number: 4309849)
6. Universal PCR plate seal (Sigma-Aldrich, catalog number: Z742420-100EA)
7. UMI_Oligo dT_T31 (100 µM) (Integrated DNA Technologies)
8. UMI_TSO6 (Integrated DNA Technologies)
9. STRT-adaptors 96 different oligos (96 well plate Integrated DNA Technologies)
10. UMI PCR (96 well plate Integrated DNA Technologies)
11. dNTP 25 mM (Thermo Fisher, catalog number: R0181)
12. 50x Advantage 2 Polymerase (Takara, catalog number: 639202)
13. Pvul (Cutsmart 10x provided) (New England Biolabs, catalog number: R3150L)
14. Dynabeads™ MyOne™ Streptavidin C1 (Thermo Fisher, catalog number: 65001)
15. Zymolyase 100T (100 mg/ml) (US Bio, catalog number: 37340-57-1)
16. RNase Zap (Thermo Fisher, catalog number: AM9780)

17. RNase Inhibitor (40 U/ml) (Takara, catalog number: 2313A)
18. RNase-nuclease free water (Thermo Fisher, catalog number: 10977035)
19. ERCC RNA spike ins (Thermo Fisher, catalog number: 4456740)
20. TAPS (Sigma-Aldrich, catalog number: T5316)
21. DMF (Sigma-Aldrich, catalog number: 74438)
22. 5x Superscript First strand buffer (Thermo Fisher, catalog number: 18064014)
23. MgCl₂ (1 M) (Thermo Fisher, catalog number: AM9530G)
24. Betaine (5 M) (Sigma-Aldrich, catalog number: 61962)
25. Superscript II (Thermo Fisher, catalog number: 18064014)
26. 10x Advantage 2 PCR Buffer (Takara, catalog number: 639202)
27. Tris (Sigma-Aldrich, catalog number: T2319)
28. Tween-20 (Sigma-Aldrich, P9416-50ML)
29. Glycerol (Sigma-Aldrich, catalog number: G5516)
30. KAPA library quantification (Kapa Biosystems, catalog number: KR0405)
31. Ampure beads (Beckman Coulter, catalog number: A63881)
32. TE 10x (Sigma-Aldrich, catalog number: T9285)
33. NaCl (Sigma-Aldrich, catalog number: S5150)
34. EDTA (Thermo Fisher, catalog number: AM9261)
35. Elution Buffer (EB) (Qiagen, catalog number: 19086)
36. PB Buffer (PB) (Qiagen, catalog number: 19066)
37. Sybergreen 2x mastermix (Invitrogen, catalog number: 4309155)
38. LNA primers (Exiqon)
39. Primers for qPCR
 - a. SOMN17 Fw_TDH3_probe: TCGTCAAGTTGGTCTCCTGG
 - b. SOMN18 Rv_TDH3_probe: GGCAACGTGTTAACCAAGT
 - c. SOMN21 Fw_ADH1_probe: TGGTGCCAAGTGTGTTCTG
 - d. SOMN22 Rv_ADH1_probe: GGCGAAGAAGTCCAAAGCTT
 - e. SOMN310 Fw_5_ERCC_00130: CGGAAAAGTACTGACCAGCG
 - f. SOMN311 Rv_5_ERCC_00130: TGCCAATGACTTCAGCTGAC
40. Optical lids for qPCR (Applied Biosystems, catalog number: 4311971)
41. DNA High Sensitivity CHIP (Agilent, catalog number: 5067-4626)
42. 1% Triton X-100 (Sigma-Aldrich, catalog number: X100-1L)
43. 100 mM DTT (Thermo Fisher, catalog number: 18064014)
44. Propidium Iodide (PI) (Sigma Aldrich, P4170-10MG)
45. Cell capturing and lysis solution (see Recipes)
46. 2x BWT Buffer (see Recipes)
47. TNT Buffer (see Recipes)

Equipment

1. 96-well plate magnet (Thermo Fisher, catalog number: 12331D)
2. 1.5-2 ml tube magnet (Thermo Fisher, catalog number: 12303D)
3. Bioanalyzer (Agilent)
4. HiSeq 2000 (Illumina)
5. FACS (BD Influx, or Aria II, nozzle 70 and 100 microns)
6. qPCR (Applied Biosystems)
7. Qubit (Thermo Fisher, catalog number: Q32854)
8. Thermocycler (any vendor)
9. Thermomixer (any vendor)
10. Multichannel pipettes (any vendor)
11. Plate centrifuge (any vendor)

Software

1. Novocraft (Novocraft Technologies Sdn Bhd, <http://www.novocraft.com/>)
2. Samtools v1.3.1 (Li *et al.*, 2009, <http://samtools.sourceforge.net/>)
3. R programming language v.3.5.0 (R Core Team, 2019, <https://www.r-project.org/>)
4. Genomic Alignments R package v.1.18.1 (Lawrence *et al.*, 2013)

Procedure

Note: If possible, generate a single-cell library preparation space in the laboratory. If not, wash the surface of the bench and all the material with RNase Zap at the beginning and end of each library. All material and reagents are handled with gloves and the standard precautions for RNA work should be taken (RNase free material and filter tips).

A. Cell growth

On the day before sorting, grow the desired pre-inoculum of your desired yeast strain in their corresponding media overnight (O/N). To profile exponentially growing cells, we recommend the initial culture not to grow over optical density $OD_{660} = 1$.

B. Cell sorting

1. The next morning (sorting day), dilute cells to $OD_{660} = 0.05$ in the corresponding media and allow for at least 2 cell divisions (3 h approximately for wild type strains) prior to cell isolation.
2. Prepare 96- or 384-well plates containing 5 μ l absolute ethanol in each well to fix cells immediately for sorting.

Notes:

- a. *During protocol optimization, we recommend using break-away plates. These plates allow*

breaking 96 well plates by rows, and thus several tests can be done using the same plate. Check with your facility the compatibility of the plates.

- b. We have obtained the same results sorting cells directly into 5 µl of “Cell capturing and lysis solution” (see below). If doing so, prepare plates right before sorting and keep them on 4 °C ice.
3. Dilute cells prior to sorting to OD = 0.05 in 3 ml of growth media and vortex vigorously to separate cell clumps.
Note: At this step, propidium iodide (PI) (4 µg/ml) can be added to check for cell viability. Adjust the culture volume to your needs. In our experience, 3 ml is enough to sort at least 10 plates.
4. At the FACS facility, filter cells with Cell Strainer Tubes (check with your facility which tubes they prefer) and put cells in the appropriate sorting tube for live single-cell sorting.
5. Check the alignment of the plate with the sorter. For example, this can be done by sorting a drop into a covered plate, and ensuring that the droplet would fall into the center of each well.
6. Sort live single yeast (propidium iodide (PI) negative) into each well of the plates, being sure to leave one well as a negative control. We index-sorted from the population using the forward and side scatter (FSC and SSC respectively).
Note: To include a positive control, sort 100 cells into one well.
7. Cover plates with Universal PCR plate seal.
8. Quick spin plates to collect cells at the bottom of each well (short spin to collect all liquid to the bottom of the wells).
9. Let the ethanol evaporate in a sterile environment (*i.e.*, sterile hood) for no more than 45 min.
10. Once the ethanol is completely evaporated, add 5 µl yeast “Cell capturing and lysis solution”. Spin down and freeze immediately.
Note: Regardless of whether the cells are sorted into ethanol or directly into “Cell capturing and lysis solution” (see Recipes), frozen plates can be stored at -80 °C for at least 6 months. ERCCs are spike in RNAs that provide an accurate measure of technical noise, while we recommend using them, they can be excluded.
11. Perform the following lysis cycle from freshly-sorted or frozen plates (Table 1).

Table 1. Temperature conditions for cell wall digestion and cell lysis

Temperature	Time	Cycles
30 °C	10 min	1
72 °C	3 min	1
4 °C	1 min	1

Note: Digestion can be extended up to 30 min.

12. Immediately proceed to add the RT reaction for first strand cDNA synthesis. Add 5 µl Reverse Transcription mix (RT mix) (Table 2).

Table 2. Master mix reagents for first strand cDNA synthesis

Reagents	Volume (μ l per reaction)
5x SuperScript Strand Buffer	2 μ l
MgCl ₂ (1 M)	0.06 μ l
Betaine (5 M)	1.6 μ l
UMI_TSO6 100 μ M	0.2 μ l
200 U/ μ l SuperScript II	0.25 μ l
RNase inhibitor (40 U/ml)	0.125 μ l
RNase-Nuclease free H ₂ O	0.765 μ l
Total	5 μ l

13. Spin down the plate and perform the following cycles (Table 3):

Table 3. Incubation temperatures for cell lysis

Temperature	Time	Cycles
42 °C	90 min	1
70 °C	15 min	1
4 °C	hold	1

14. Add 15 μ l PCR mix for library amplification to each well (Table 4).

Table 4. Master mix reagents for library amplification

Reagents	Volume (μ l per reaction)
10x Advantage 2 PCR buffer	2.5 μ l
dNTPs (25 mm)	0.4 μ l
UMI_PCR (10 μ M)	1.2 μ l
50x Advantage 2 polymerase mix	0.5 μ l
H ₂ O	10.4 μ l
Total	15 μ l

15. Spin down the plate and do the following cycles (Table 5):

Table 5. PCR cycling conditions for library amplification

Temperature	Time	Cycles
95 °C	1 min	1

95 °C	20 s	
58 °C	4 min	5
68 °C	6 min	
95 °C	20 s	
64 °C	30 s	9
68 °C	6 min	
95 °C	30 s	
64°C	30 s	7
68 °C	7 min	
72 °C	10 min	1
4 °C	hold	1

Note: This is a safe stopping point (at 4 °C O/N, or frozen -20 °C for 1-2 months).

16. (Optional) Dilute the amplified library 1:20 and check the percentage of positive cells from Step B15 (Figure 1; Tables 6 and 7).

Note: Use primers against a housekeeping gene (we use TDH3 but we have obtained same results with ADH1) to measure the number of positive cells per well. Use primers against ERCC as a positive control for amplification that should be even across all reactions. This step is especially useful during the protocol set up as it allows inspecting the samples/libraries before moving forward (check for the number of positive libraries). See [Table S1](#) for primer sequences.

Table 6. Master mix for qPCR assessment

Reagent	Volume (μl per reaction)
Sybergreen 2x	2.5 μl
Primer mix	0.125 μl
H ₂ O	1.375 μl
DNA (diluted)	1 μl
Total	5 μl

Note: Primer mix refers to the mixture of Fw and Rv primers (10 μM each) diluted in TE 1x.

Table 7. PCR cycling conditions for qPCR

Temperature	Times	Cycles
-------------	-------	--------

95 °C	10 min	1
95 °C	30 s	
58 °C	30 s	40
72 °C	30 s	
95 °C	15 s	
60 °C	15 s	melting curve
95 °C	15 s	

Primer sequences for qPCR:

SOMN17 Fw_TDH3_probe: TCGTCAAGTTGGTCTCCTGG

SOMN18 Rv_TDH3_probe: GGCAACGTGTTCAACCAAGT

SOMN21 Fw_ADH1_probe: TGGTGCCAAGTGTGTTCTG

SOMN22 Rv_ADH1_probe: GGCAGAAGAAGTCCAAAGCTT

SOMN310 Fw_5_ERCC_00130: CGGAAAAGTACTGACCAGCG

SOMN311 Rv_5_ERCC_00130: TGCCAATGACTTCAGCTGAC

Note: A good plate will have around 70% positive wells considering the Ct values of the housekeeping gene. Plates with less than 50% positive cells are rare. To determine if low efficiency is due to sorting or due reaction efficiency, perform a qPCR using a 1:10 dilution of the cDNA library against ERCCs. Failure to amplify ERCCs, or uneven amplification (represented by wildly different Ct values), is indicative of incorrect library preparation. In the case of low number of positive cells per plate, you can generate a new plate by combining positive from different plates into a new plate before proceeding to tagmentation and try to improve sorting efficiency.

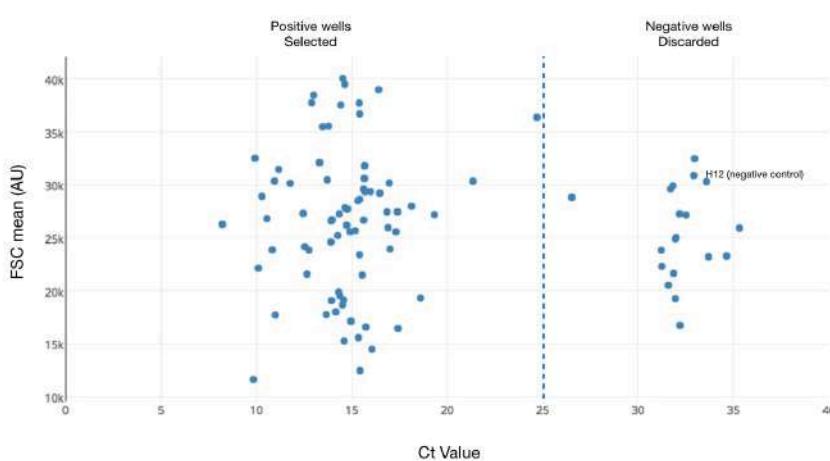


Figure 1. Representative example of Ct Values obtained by qPCR. The scatter plot represents the cycle amplification (Ct value) of a yscRNA-seq 96-well plate. Each dot represents the value obtained using a yeast housekeeping gene (*TDH3*, x-axis) as a function of the index-sorting value for cell size (Forward Scatter (FSC), y-axis). Dotted line (Ct > 25) displays the

threshold used to discriminate positive and negative wells. The label displays the Ct value for the not sorted well H₁₂ (which is used as a negative control).

17. Add 15 µl of room temperature equilibrated and well homogenized Ampure XP beads (1:0.6 sample/ bead ratio) to each well.

Note: Do not increase the volume of beads in the purification step above the 1:1 ratio. A less-than-standard amount of beads ensures that primer dimer carryover is minimal.

18. Mix by pipetting up and down ten times, or until the solution appears to be homogeneous. Transfer solutions to a 96-well plate with compatible magnet stand.

19. Incubate the mixture for 10 min at room temperature to let the DNA bind to Ampure XP beads.

20. Place the 96-well plate on the magnetic stand for 5 min, or until the solution is clear and beads have been collected.

21. While samples are on the magnet, carefully remove the liquid without disturbing the beads.

22. Wash magnet-bound beads with 200 µl of 80% (vol/vol) ethanol solution. Incubate the samples for 30 s and then remove the ethanol with the tube in the magnet, do not overdry the beads.

Note: It is important that the ethanol solution is freshly prepared every time, as ethanol absorbs moisture from the environment, thus changing the final concentration. Read and follow the manufacturer's instructions.

23. Repeat the ethanol wash one more time (repeat Steps B20-B22).

24. Remove any trace of ethanol and let beads dry completely by leaving the plate at room temperature for 5 min or until ethanol evaporates.

Note: Cover the plate during this step or protect it from any possible source of contamination or airflows.

25. Once there is no ethanol left, elute dscDNA libraries from Ampure XP beads with 16.5 µl elution buffer at room temperature (EB buffer Qiagen).

26. Remove the plate from the magnet and mix vigorously by pipetting up and down three times or until the solution becomes homogeneous.

27. Place the plate on a magnetic stand and leave it for 2 min, or until the solution appears clear.

28. Recover 15 µl of supernatant from each well and transfer to a new plate. Label the plate correctly, as it will be stored.

Safe stopping point: cDNA libraries can be stored at -20 °C before proceeding to tagmentation for up to 2 weeks.

C. Full-length cDNA library quality check

1. Run 1 µl of dscDNA libraries (Step B28) to check the size distribution and estimate of concentrations using a High Sensitivity DNA ChIP (2100 Bioanalyzer). If available, use the qPCR results from Step B16 to guide the selection of wells for Bioanalyzer (Figure 2).

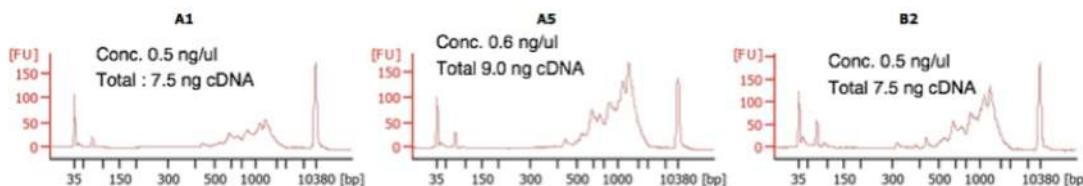


Figure 2. Representative Bioanalyzer traces of full-length cDNA obtained with yscRNA-seq (Step C1). cDNA libraries obtained from step (Step B28) were run on a DNA High Sensitivity CHIP (Agilent 5067-4626) for validation. Library concentration was also measured by Qubit High Sensitivity (Thermo Fisher). Left panel (A1) represents the lower limit of library quality that we sequenced while middle (A5) and right (B2) panel represent average libraries (Figure adapted from Nadal-Ribelles et al., 2019).

Adaptor annealing: In order to generate cell-barcoded libraries, Tn5 needs to be loaded with double stranded DNA (dsDNA) adapters. To do so, 96 different dsDNA adapters need to be annealed.

2. Thaw the plate with the 96 STRT barcodes (100 μM) in ice.
3. In a new plate, mix 5 μl UMI-TN5-U (100 μM) and 5 μl UMI-TN5_1 to 96 (μM) in TE 1x to final concentration 50 μM (each), a 1:1 dilution.
4. Anneal primers by heating the mix at 95 °C for 3 min and gradually cool down to room temperature (0.5 °C/s). Label the plate as “Annealed cell-specific adapters” plate.
Note: Label plates with the amount of annealed primer and the annealing date. Plates can be prepared in advance and stored at -20 °C for up to six months. The amount of annealed adaptors depends on the number of plates that need to be tagmented and the frequency of usage.
5. To load cell specific adapters to Tn5, prepare a new plate and label it as “loaded Tn5”. In each well, mix the following reagents. We recommend to make a mix with all reagents and add annealed cell-specific adapters individually (Table 8).

Table 8. Master mix to generate 10x transposome

Reagent	Volume	Observation
annealed cell-specific adaptors 50 μM adapter (96 different) from step C4	1.25 μl	add individually
80% Glycerol	6.25 μl	premix
50 μM Tn5 transposase	1.25 μl	premix
Nuclease-free water	1.25 μl	premix
Total	10 μl	

6. Load Tn5 by incubating at 37 °C for one hour and immediately cool to 4 °C. Freeze the “loaded Tn5” plate at -20 °C.

Note: Loaded Tn5 plate can be safely stored for 1-2 weeks at -20 °C. Caution, store the plate immediately after use leaving the loaded plate on ice 4 °C will significantly reduce Tn5 activity

and will result in inefficient fragmentation.

Fragmentation

7. Prepare the following mix in a new plate in ice (Table 9).

Table 9. Master mix for library fragmentation

Reagent	Volume (μ l per well)
Harvested DNA	6 μ l
Nuclease-free water	8 μ l
2x TAPS buffer	2 μ l
100% DMF	2 μ l
10x Transposome	2.0 μ l
Total	20 μl

8. Incubate at 55 °C for 5 min and 3 min at 85 °C to inactivate Tn5, then cool to 4 °C in a thermocycler. Fragmentation time depends on Tn5 purification efficiency and activity. We have observed that the enzyme loses activity over time. Titration of each batch of Tn5 is strongly suggested by fragmenting the same amount cDNA (Steps B28 and C1) with increasing concentration of loaded Tn5 (Step C6) and check fragmentation profile in a Bioanalyzer.
9. Prepare a 1:20 dilution of MyOne C1 Streptavidin per each sample.
10. Wash MyOne beads 2 x with 2x BWT buffer and dilute with 20x more volume than the original volume of beads with 2x BWT.
Example: 20 μ l beads for 20 samples will be finally diluted with 400 μ l 2x BWT.
11. Add 20 μ l of diluted MyOne C1 Streptavidin beads (step C10) to each well and incubate at RT for 5 min at room temperature.
12. Pool all samples per plate (up to 96) into a single collecting tube (1.5 or 2 ml).
13. Place collecting tube in magnetic rack and allow enough time for the solution to be completely clear.
14. While on the magnet, wash beads once with 100 μ l of TNT buffer do not mix the beads.
15. While on the magnet, wash MyOne C1 Streptavidin once with 100 μ l PB Buffer and discard the supernatant.
16. While on the magnet, wash beads 3 x with 100 μ l TNT buffer again and the discard supernatant.

Remove 3' ends

17. Add 100 μ l of the following mix to the washed beads from Step C14 (Table 10).

Table 10. Mastermix for 3' end removal

Reagents	Volume (μ l per reaction)
Cutsmart buffer 10x	100 μ l
Pvul-HF enzyme	2 μ l
Nuclease free H ₂ O	88 μ l
Total	20 μ l

18. Incubate mix for 1 h at 37 °C in a thermomixer. Mix every 2 min for 30 s at 1,000 rpm, to avoid bead sedimentation.
19. Wash beads 3 x with 100 μ l of TNT buffer.

Elute single stranded cDNA

20. Resuspend in 30 μ l Nuclease-free water.
21. Incubate 10 min at 70 °C, 850 rpm mix in a thermomixer.
22. Immediately bind beads to the magnet and transfer the supernatant to a new tube, which contains the single strand cDNA library in the supernatant (the other strand remains bound to the streptavidin beads).

Single-strand cDNA cleanup

23. Add 54 μ l of room temperature Ampure XP beads to 30 μ l ssDNA library.
24. Incubate for 10 min at RT.
25. Bind beads to the magnet for 1 min or until the solution is completely clear and discard supernatant (keep the beads).
26. Wash once with 200 μ l fresh 80% ethanol for 20-30 s. Perform this step with the beads bound to the magnet.
27. Air dry beads for approximately 2 min.
28. Resuspend in 30 μ l EB buffer and incubate for 5 min at RT.
29. Bind beads for 2 min, or until the solution is clear, and transfer the supernatant to a new tube.

D. Assess library concentration

1. Set up a KAPA quantification reaction with a 1:100, 1:1,000 and 1:10,000 dilutions of the eluted cDNA library (Step C27). All reagents except for your DNA library are provided in the kit (Table 11).

Note: This kit can be substituted by your favorite quantification method or by a qPCR using P5-P7 primer pairs with known standards (PhiX is strongly recommended) SYBRGreen 2x mastermix.

Table 11. Master mix for qPCR library quantification

Reagents	Volume (μ l per reaction)
KAPA SYBR® FAST qPCR Master	12 μ l
Mix containing Primer Premix	
PCR-grade water	4 μ l
Diluted library DNA or DNA Standard	4 μ l
Total	20 μ l

2. qPCR cycling conditions for KAPA and homemade Sybergreen (Table 12).

Table 12. PCR cycling conditions for qPCR

Temperature	Time	Cycles
95 °C	5 min	1
95 °C	30 s	30
60 °C	45 s	
4 °C	hold	

3. Use the qPCR to calculate library quantification using the template provided by KAPA biosystems or the instructions provided from your manufacturer.

Note: We have used KAPA, NEB and homemade systems with similar results.

4. Set up a separate PCR to run a bioanalyzer to determine the final size distribution. Prepare the following mix, one separate reaction per each library to be loaded (Table 13).

Table 13. Master mix for to assess library size after tagmentation

Reagents	Volume(μ l per reaction)
KAPA SYBR Q-PCR Mastermix ABI Prism	10 μ l
Primers mix (10x)	2 μ l
Water	6 μ l
DNA without dilution	2 μ l
Total volume	20 μ l

5. Run the same PCR as in Step D2 but for 11 cycles.
6. Run 1 μ l into a High sensitivity DNA CHIP to obtain an average library size based on the Bioanalyzer profile (Figure 3).

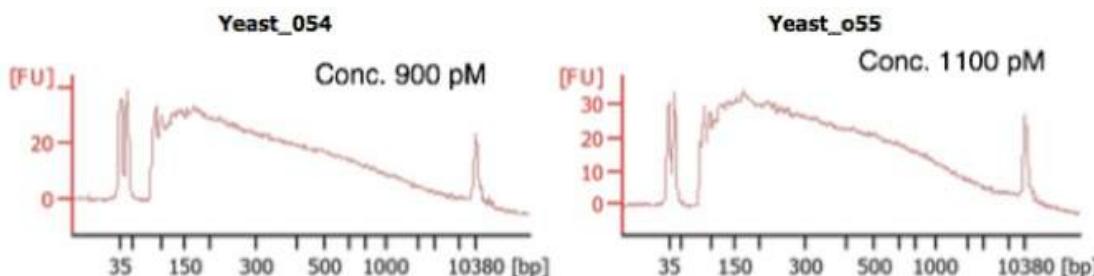


Figure 3. Representative Bioanalyzer traces obtained from yscRNA-seq. Two representative samples obtained from approximately 80 cells. Concentration of each library is shown and was determined by qPCR (Step D3). Figure from Nadal-Ribelles et al., 2019.

7. Sequence the library on the HiSeq 2000 High output using the custom *Read 1* primer and UMI-TN5-U as the *Index read* primer.
8. To run the libraries on the HiSeq rapid run, use lock nucleic acid (LNA) primers. Spike in the primer at 0.5 μ M.

Index 1 primer into HP8

Read 1 primer into HP9

Notes:

- a. Double-check this information with your sequencing kit/instrument and/or sequencing core facility.
- b. For a High Output Run, custom primers are required as well, but without LNA due to differences in sequencing chemistry.

UMI_PCR_read1: +GAATGA+TACGGCG+ACCA +CCGA+T – custom 250 nmole. DNA oligo, HPLC Purification

Index1: CTGT+CT+CTT+ATA+CA +CA+TCTGA+CG+C – custom 250 nmole DNA oligo, HPLC Purification

9. Load around 8-14 pmol of each library per lane. Libraries are single-stranded DNA, thus, no denaturing is required.

Note: Once the protocol is optimized, there is no need to run a PhiX control, if PhiX is loaded take into consideration a denaturing step for the double-stranded PhiX control, which is not required for yscRNA-seq libraries.

A schematic representation of the plate processing is shown in Figure 4 as a reference.

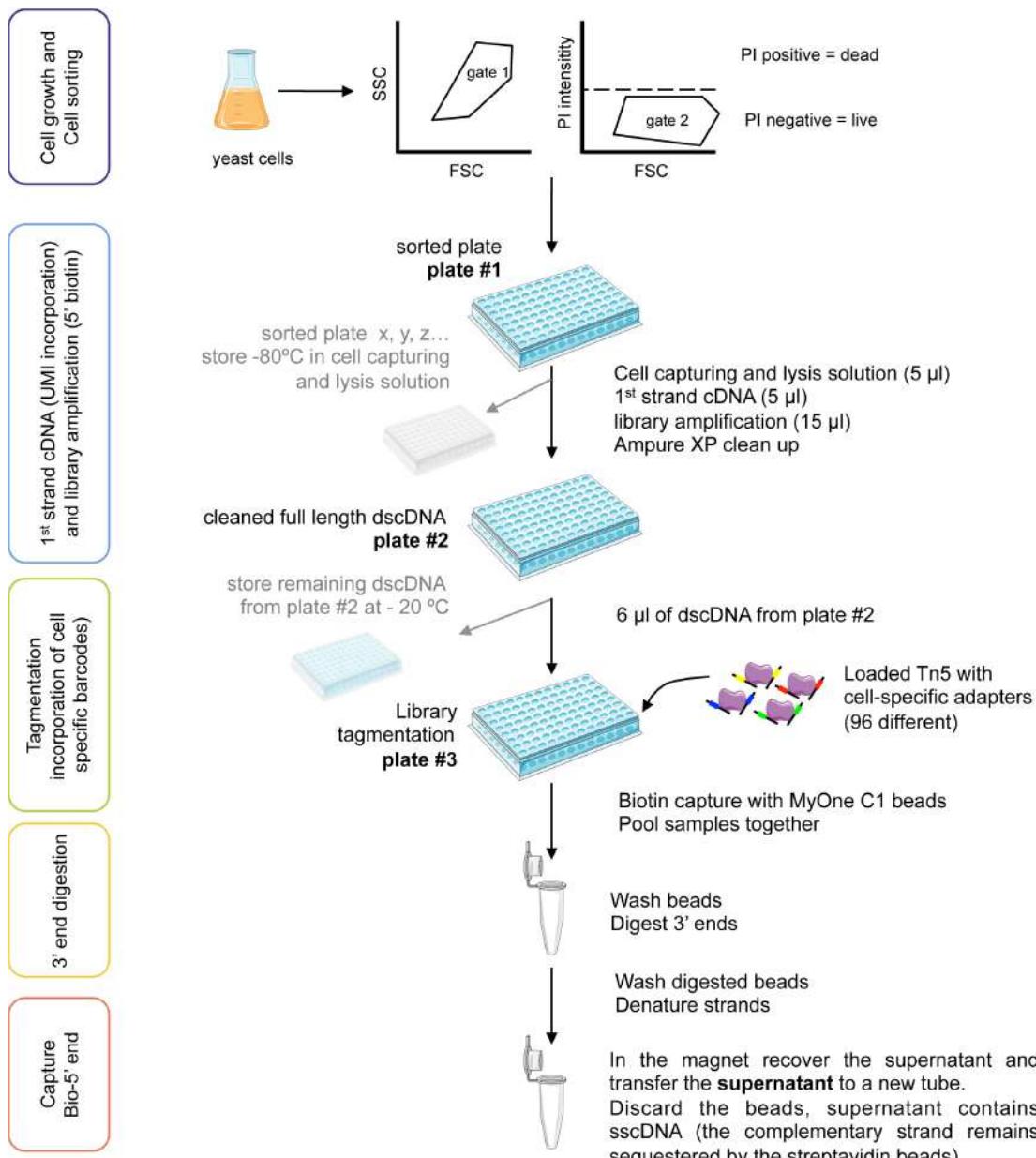


Figure 4. Schematic representation of yscRNA-seq. Images from Smart Medical server (Les Laboratoires Servier, [SMART Servier Medical Art.](#)).

Data analysis

The initial steps of yscRNA-seq data analysis are very similar to those applied for bulk RNA-seq (Conesa *et al.*, 2016), but with certain particularities. YscRNA-seq generates two reads per sequenced cDNA molecule. The first read (Index 1) contains the cell-specific barcode that associates a given molecule with a specific cell. The second read (Read 1) contains the UMI, and maps to the TSS of a specific transcript, allowing for absolute molecule-counting and transcript identification, respectively. To properly estimate gene expression from yscRNA-seq data, both reads have to be computationally associated to their respective references. The former association is

performed by default in Illumina's *BaseSpace* demultiplexing tool. This generates individual FASTQ files (one per each yeast cell) that are the starting point of yscRNA-seq analysis. After this, we further pre-process the FASTQ files to identify the UMIs and perform the alignment and quantification to obtain the gene expression table (Figure 5). Here, we detail the steps that we follow for analyzing yscRNA-seq data, starting from FASTQ files:

Note: The analysis pipeline was executed in a Linux 64 bit machine with 32 Gb of RAM and 6 CPUs. We recommend to have at least 12 Gb of RAM. A machine with several computing cores is also preferable for speeding up the processes.

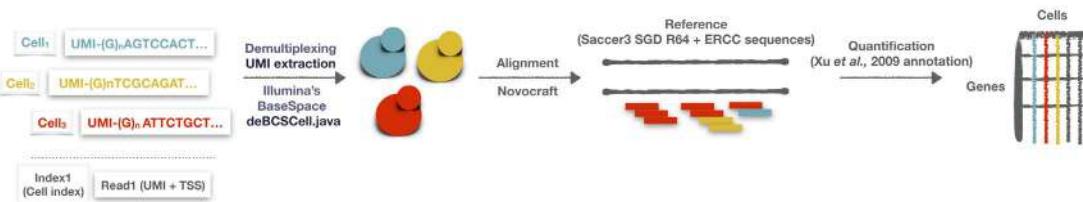


Figure 5. Schematic representation of analysis pipeline of yscRNA-seq

A. Read pre-processing

To extract the UMI from the beginning of the reads, we used a custom JavaScript program ([deBCSCell](#)).

Command: `$ java -cp directoryOfdeBCSCell rootNameOfdeBCSCell f1=fastqFile BL=""`

This script performs the following actions:

1. Extracts the first 6nt (UMI) and incorporates them into the read name for later use.
2. Extracts and counts N's and G's following the UMI, up to 14. The read is discarded if it contains 14 or more N's or G's.

B. Alignment

*Note: In our case, the reference sequences (referenceSequencesName.fa) are a combination of the *S. cerevisiae* genome (Saccer3, SGD R64 version; www.yeastgenome.org) and the ERCC control sequences. The ERCC control sequences provided by the manufacturer do not include the restriction sites used during the cloning of the sequences downstream of the T7 promoter. Since yscRNA-seq is TSS-specific, this fact considerably decreases the mapping rate due to misalignments at the beginning of the read. To overcome that, we generated a new ERCC reference that includes these sequences. These modified references could be downloaded from the Gene Expression Omnibus (under [GSE122392](#) accession number).*

1. *In our case, we have sequenced each library to a median depth of ~720,000 reads. We assessed, with down-sampling, that this depth is substantially above the needed to obtain a*

high resolution of the yeast transcriptome. In fact, at ~500,000 reads per cell in the number of transcripts detected saturation is reached (Supplementary Figure 2C [Nadal-Ribelles et al., 2019]). In addition, we observe a median of ~74% uniquely mapped reads for all sequenced libraries. These values are useful reference values using this protocol.

2. We used Novocraft for the alignment of the reads, but open-source aligners such as HISAT2 (Kim et al., 2015) or STAR (Dobin et al., 2013) can be used without obtaining major differences in the results.

Reads were aligned with Novocraft (<http://www.novocraft.com/>) using default parameters.

1. Create the index for Novocraft using novoindex command:

```
$novoindex referenceSequencesName.nix referenceSequencesName.fa
```

2. Align reads using novoalign command:

```
$novoalign -f fastqFileUMIprocessed.fastq -d referenceSequencesName -o SAM |
```

3. Convert to BAM format and sort it using Samtools view and Samtools sort: (piped from the previous command)

```
| samtools view -bs - | samtools sort - fileName.sorted.bam.
```

C. Quantification

Although we are aware of the existence of public software for quantifying UMI-based single-cell data (such as UMI-tools [Smith et al., 2017]), we performed quantification using R custom scripts to have full flexibility and control over data inspection and data filtering. The starting point of the quantification is the sorted BAM files obtained in the previous step, one for each sequenced cell.

1. Process the BAM files.

Script: `readAlignUMI.R`. `$Rscript readAlignUMI.R`

Each BAM file is loaded into R using the *Genomic Alignments* package. Then, low quality mapping reads (MAPQ < 30) and reads that map with soft-clipping in the 5' end are filtered out. Finally, reads that map to the same position and that have the same UMI are grouped for collapsing them in the next steps.

2. Process UMI grouped data.

Script: `bard2rds.R`. `$Rscript bard2rds.R`

At this step, UMIs supported with less than 3 reads are filtered out since they could represent sequencing errors. Then, different UMIs that map to the same genomic position are grouped. Different UMIs mapping to the same position represent different molecules of the same transcript. This information is stored into a *RangedData* object.

3. Overlap with annotation and count table generation.

Script: txAnno.R (#link). \$Rscript txAnno.R.

If there is overlap, each molecule is assigned to a genomic feature from an annotation. In this study, we used the annotation from (Xu *et al.*, 2009) since it includes a comprehensive categorization of genes into different classes (coding, CUTs, SUTs and others). The output obtained is an absolute gene expression table with genes as rows and cell as columns.

D. (Optional) Filtering

To avoid the presence of low-quality cells (dead, stressed or apoptotic), we decided to keep only those with more than 500,000 sequenced reads, and those in which we detect more than 1,000 different transcripts. In our case, this filter eliminates cells with a high ratio of mitochondrial RNA, which has been associated with low quality cells (Ilicic *et al.*, 2016). However, this filter is not imperative and could be tuned depending on the type of experiment and on the biological question under study.

Recipes

Note: All reagents and water used must be nuclease-free and only used for single cell protocols.

1. Cell capturing and lysis solution (Table 14)

Table 14. Composition of “Cell capturing and lysis solution”

Reagents	Volume (μl per reaction)
1% Triton X-100	0.05 μl
UMI_Oligo dT_T31 (100 mM)	0.2 μl
dNTP 25 mM	0.68 μl
100 mM DTT	0.5 μl
Zymolyase 100T (100 mg/ml)	0.6 μl
RNase Inhibitor (40 U/ml)	0.12 μl
RNase-Nuclease free H ₂ O	2.769 μl
ERCC (1:1,000,000) (5,000 molecules)	0.081 μl
Total	5 μl

2. 2x BWT Buffer
 - 10 mM Tris-HCl pH 7.5
 - 1 mM EDTA
 - 2 M NaCl
 - 0.02% Tween-20
3. TNT Buffer
 - 20 mM Tris pH 7.5
 - 50 mM NaCl
 - 0.02% Tween

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Competing interests

The authors declare no financial or non-financial interests.

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Production of Quantum Dots-containing Influenza Virus Particles for Studying Viral Uncoating Processes

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[Abstract] The genome of influenza A virus (IAV) comprises eight pinlike genomic segments called vRNPs enclosed in viral capsid. During infection, uncoating is the key step for viral replication and represents an antiviral therapeutic target, but it is difficult to observe the transient and dynamic event in detail. Here, we report a protocol for production of quantum dots-containing influenza virus particles by encapsulating quantum dot-conjugated vRNPs during viral assembly. These labeled virions can be used for monitoring viral trafficking in real time and studying viral uncoating processes.

Keywords: Influenza A virus, vRNP, Quantum dots, Viral assembly, Uncoating

[Background] Influenza A virus (IAV) is one of the most important human pathogens. Viral entry before virus-endosome fusion has been widely studied by various methods, including single-particle tracking. However, the viral uncoating process following endocytosis remains elusive. Robust live imaging strategies that offer high temporal and spatial resolution are essential to study this dynamic and transient event. However, the influenza RNA genome is intolerant to insertion of large genetic materials, and rescue of viruses using fluorescent protein fused to viral genomic core proteins have been limited (Lakdawala *et al.*, 2014). This has delayed progress in the field of influenza-uncoating and vRNPs-dynamics live-imaging studies. Although purified vRNPs have been dye-labeled and microinjected into cells to track their intracellular transportation and nuclear import process in living cells in real time (Babcock *et al.*, 2004), this method cannot capture the real infection process of incoming virions or determine vRNP behaviors during uncoating. Fluorescence *in situ* hybridization (FISH) and colocalization analyses with single-molecule resolution have also been used to study the behavior of vRNPs in infected cells (Chou *et al.*, 2013), but the fixation procedures are not compatible with imaging vRNP dynamics in living cells. Semiconductor quantum dots (QDs) have unique optical properties, such as remarkable brightness and superior photostability, and are well-suited to single-particle tracking of viral infection. Single-particle tracking of virions with QDs-labeled genetic material provides an opportunity for tracking viral uncoating and post-uncoating genetic behaviors in real time. As a subunit of polymerase complex, PA protein participates in the composition of vRNP-genetic core of IAV. In PNAS, Qin *et al.* (2019) developed a nanotechnology that labels IAV viral ribonucleoprotein complexes (vRNPs) with QDs. Briefly, the biotin acceptor peptide (AP tag) was genetically fused to the PA protein to construct a recombinant PR8 strain. The rescued rPR8-PA-AP virus was then cultured in BirA-expressing MDCK cells in the presence of biotin. Streptavidin-coated QDs (SA-QDs) were introduced into these cells by

lipofection to allow noncovalent binding of the QDs to biotinylated PA. QDs-containing influenza virus particles were thus generated. This approach will advance the mechanistic understanding of influenza virus uncoating using live fluorescence microscopy.

Materials and Reagents

A. Materials

1. 1.5 ml graduated microcentrifuge tubes (QSP, catalog number: 509-GRD-Q)
2. 2 ml graduated microcentrifuge tubes (QSP, catalog number: 508-GRD-Q)
3. SW32 Ultra-Clear Tubes (Beckman Coulter, catalog number: 344058)
4. SW41 Ultra-Clear Tubes (Beckman Coulter, catalog number: 344059)
5. 0.1-10 µl pipette tips (QSP, catalog number: T104RS-Q)
6. 10-200 µl pipette tips (QSP, catalog number: TTW110RS-Q)
7. 100-1250 µl pipette tips (QSP, catalog number: T112NXLRS-Q)
8. 12-well plates (Corning Costar, catalog number: 3513)
9. 6-well plates (Thermo Scientific, catalog number: 140675)
10. V-bottom 96-well plates (Solarbio, catalog number: YA0590)
11. Scalpel Blade (Hong Yue, catalog number: GC-HY00388)
(link: <https://www.casmart.com.cn/product-details/page/103/11044449>)
12. 100 mm Nunc EasYDishes (Thermo Scientific, catalog number: 150464)
13. 75 cm² Nunc EasYFlasks (Thermo Scientific, catalog number: 156472)
14. 25 cm² Nunc EasYFlasks (Thermo Scientific, catalog number: 156340)
15. 0.45 µm filter (Millex-HV, catalog number: SLHU033RB)
16. Filter paper (Biorad, catalog number: 1703932)
17. 3 ml Pasteur pipettes (Nest, catalog number: 318314)
18. 1 ml syringes (Gemtier, catalog number: JT-001)
19. 10 ml syringes (Gemtier, catalog number: JT-010)
20. 15 ml centrifuge tubes (Nest, catalog number: 601052)
21. 50 ml centrifuge tubes (Nest, catalog number: 602052)

B. Cell lines

1. 293T cells (Boster, catalog number: CX0008; ATCC, catalog number: ACS-4500)
2. MDCK cells (Boster, catalog number: CX0206; ATCC, catalog number: CRL-2936)

C. Plasmids

1. Eight plasmids (pHW2000) each containing individual cDNA segment of the influenza virus strain A/Puerto Rico/8/34 (H1N1) (Hoffmann et al., 2000)
2. pcDNA3.1(+)-BirA

Note: All plasmids are available from our lab. BirA is an enzyme that catalyzes ligation of Biotin and

AP tag.

D. Primers

Linearized Vector-F: GTGCTACTATTGCTATCCATACTGTCC

Linearized Vector-R: ACATCTTCTCCAATCTCATCGAGCTCTATC

Insert-F1: GAAAATCGAATGGCACGAATAGTCTAGAAAAGCTGCTTATCGTCAGG

Insert-R1: TGGACAGTATGGATAGCAAATAGTAGCAC

Insert-F2: GATATCTCGAACGCTCAGAAAATCGAATGGCACGAATAGTCT

Insert-R2: TGGACAGTATGGATAGCAAATAGTAGCAC

Insert-F3: GAGTGGTCAGGAGGCCGGTCTGAACGATATCTCGAACGCTCAGAAAATCGAAT

Insert-R3: TGGACAGTATGGATAGCAAATAGTAGCAC

Insert-F4: GATAGAGCTCGATGAGATTGGAGAAGATGT

Insert-R4: GTTCAGACCGCCCTCTGAACCCTCAATGCATGTGTAAGGAAGGAGTTG

Insert-F5: GATAGAGCTCGATGAGATTGGAGAAGATGT

Insert-R5: TGGACAGTATGGATAGCAAATAGTAGCAC

PA-F: AGCGAAAGCAGGTACTGATCCAAATG

PA-R: AGTAGAAACAAGGTACTTTTGACAGTATG

Note: All primers were synthesized by Sangon Biotech.

E. Reagents

1. SPF embryonated chicken eggs (Merial-vital)
2. Competent cells DH5 α (TIANGEN, catalog number: CB101-01)
(Link: <http://www.tiangen.com/?productShow/t1/6/id/160.html>)
3. 70% ethanol (Amresco, catalog number: E505)
4. 2 kb Plus DNA ladder (TransGen, catalog number: BM121)
5. Ampicillin (MDBio, catalog number: 69-52-3)
6. SA-QD625 (Invitrogen, catalog number: A10196)
7. KOD FX DNA polymerase enzyme (TOYOBO, catalog number: KFX-101)
8. T4 DNA polymerase (NEB, catalog number: M0203S)
9. 50x TAE buffer (Sangon Biotech, catalog number: B548101-0500)
10. Agarose G-10 (BIOWESTE, catalog number: 111860)
11. Omega Cycle-Pure Kit (OMEGA, catalog number: D6492-01)
12. Omega Viral RNA Kit (OMEGA, catalog number: R6874-02)
13. Omega gel extraction kit (OMEGA, catalog number: D2500-02)
14. Omega Plasmid Mini kit (OMEGA, catalog number: D6943-02)
15. One Step SYBR PrimeScript PLUS RT-PCR Kit (TaKaRa, catalog number: RR096A)
16. DMEM (Gibco, catalog number: 11965118)
17. MEM (Genom, catalog number: GNM41500)
18. DMEM Powder (Gibco, catalog number: 12100061)

19. Low gelling temperature Agarose (Sigma-Aldrich, catalog number: A9045)
20. FBS (Gibco, catalog number: 1787602)
21. 7.5% BSA (Solarbio, catalog number: H1130)
22. TPCK-trypsin (Sigma-Aldrich, catalog number: T1426)
23. 1x PBS (Gibco, catalog number: 10010049)
24. Lipofectamine 2000 (Invitrogen, catalog number: 11668019)
25. Biotin (Sigma-Aldrich, catalog number: B4639)
26. Sucrose (HUSHI, catalog number: 10021418)
(Link: <https://www.reagent.com.cn/goodsDetail/94d364146ee0495d955089dbd33dade3>)
27. NaCl (HUSHI, catalog number: 10019318)
(Link: <https://www.reagent.com.cn/goodsDetail/d8517dae2fd14870aeeaddbb8b0a13f4>)
28. Tryptone (OXOID, catalog number: LP0042)
29. Yeast extract (OXOID, catalog number: LP0021)
30. LB medium with 100 µg/ml ampicillin (see Recipes)
31. LB agar plate with 100 µg/ml ampicillin (see Recipes)
32. Cell culture medium (see Recipes)
33. Infection medium (see Recipes)
34. 0.5% Chicken red blood cell (RBC) (see Recipes)
35. 2x plaque medium (see Recipes)
36. 1.6% agar (see Recipes)
37. Sucrose solution (see Recipes)

Equipment

1. Eppendorf Pipettes (0.1-10 µl, 10-200 µl, 100-1250 µl)
2. 6 W UV transilluminator (UVP, model: UVLM-26)
3. Rotating incubator (FUMA, model: QYC-200)
4. Centrifuge (Eppendorf, model: 5810R)
5. Ultracentrifuge (Beckman, model: OPTIMA XE-100)
6. Water bath (YIHENG17, model: DK-8D)
7. Beckman SW32 rotor
8. Beckman SW41 rotor
9. PCR machine (GnenAmp, PCR System 9700)
10. 100 kV Transmission electron microscope (Hitachi, model: H-7000 FA)

Procedure

A. Construction of pHW2000-PA-AP tag

The PA-AP segment was constructed by inserting a sequence encoding the short linker GSGG (Nucleotide sequence: GGTCAGGAGGC), a 15-amino acid biotin AP tag (Nucleotide sequence: GGTCTAACGATATCTCGAGCTCAGAAAATCGAATGGCACGAA), and a duplication of 165 base pairs of the 3' end of the PA-coding region after the PA ORF (stop codon deleted) using one-step sequence and ligation-independent cloning method (Jeong *et al.*, 2012). Schematic of pHW2000 PA-AP tag is shown in Figure 1.

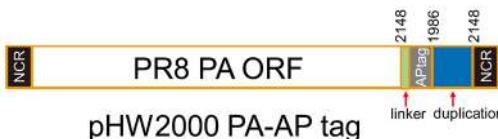


Figure 1. Schematic of the constructed plasmid pHW2000 PA-AP

1. PCR amplification and gel purification of linearized vector fragment
 - a. The linearized vector fragment (pHW2000: 2,980 bp, partial PA sequence: 1,368 bp) is amplified using the KOD FX DNA polymerase enzyme in a PCR machine, according to the manufacturer's protocol. Reaction system (Table 1) and parameters (Table 2) are shown as follows:

Table 1. The PCR reaction mixture used for the linearized vector fragment

Reagent	Volume (μ l)
DNA	1 (50 ng)
Linearized Vector-F primer	1
Linearized Vector-R primer	1
2x KOD buffer	25
KOD	1
dNTP	4
H ₂ O	17
Total	50

Table 2. The PCR reaction parameters used for the linearized vector fragment

Procedure	Temperature (°C)	Time (min)
1	95	5
2	95	0.5
3	57	0.5
4	68	4.5 (1 kb/min)
2-4	95-57-68	30 cycles
5	68	10
6	4	$+\infty$

- b. Prepare a 1% agarose-TAE gel (Add 3 μ l of 1 mg/ml EB into 30 ml solution for a gel).
 - c. Add 1/10 volume of 10x DNA loading dye to each PCR sample and load 50 μ l reaction onto the gel. In addition, load the 2 kb Plus DNA ladder for size verification.
 - d. Run the gel in 1x TAE until the bands of the loading dye run to approximately three quarters of the way down the gel.
 - e. Visualize the PCR product on a UV transilluminator. Cut the ~4,300 bp band using a clean blade. Place the gel slice into a clean 1.5 ml microcentrifuge tube.
 - f. Purify the PCR product using the Omega gel extraction kit as per the manufacturer's protocol. Elute the linearized vector fragment PCR product with 50 μ l elution buffer.
2. PCR amplification and gel purification of insert fragment
 - a. Acquire the insert fragment (partial PA sequence: 895 bp, linker-AP tag: 45 bp, and duplicated packaging sequence of PA gene: 205 bp) through a 5-steps PCR procedure. For a flow chart of these steps see Figure 2.
 - i. Firstly, amplify duplicated packaging fragment from PA gene with Insert-F1 and Insert-R1 primers.
 - ii. Then duplicated packaging fragment is used as the template to amplify AP tag-duplicated packaging fragment with Insert-F2 and Insert-R2 primers.
 - iii. The same linker-AP tag-duplicated packaging fragment can be acquired in the third step.
 - iv. Partial PA fragment is acquired from PA gene using Insert-F4 and Insert-R4 primers in the fourth step.
 - v. Finally, the linker-AP tag-duplicated packaging fragment and partial PA fragment are fused together and amplified using Insert-F5 and Insert-R5 primers by overlap extension PCR.

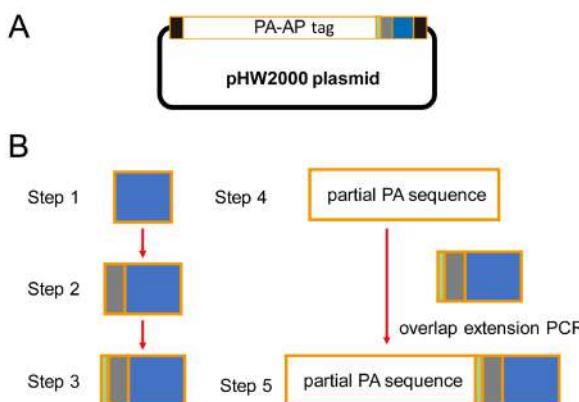


Figure 2. Flow chart diagram of the insert fragment acquisition. A. Schematic diagram of pHW2000 PA-AP. B. Flow chart diagram of the 5-steps PCR procedure.

- b. PCR amplification and gel purification are performed as described above with reference to linearized vector fragments. Elute the Purified PCR product (~1,200 bp) with 50 μ l elution buffer.

3. One-step sequence- and ligation-independent cloning
 - a. Mix the linearized vector fragment and insert fragment at a molar ratio of 1:2 in a 1.5 ml tube. Reaction system is shown in Table 3.

Table 3. Reaction system of One-Step sequence- and ligation-independent cloning

Reagent	Stock concentration	Volume added	Final concentration
Linearized vector	100 ng/μl	3.7 μl	37 ng/μl
Insert	100 ng/μl	2 μl	20 ng/μl
10x BSA		1 μl	1x
10x NEB buffer		1 μl	1x
H ₂ O		Up to 10 μl	

- b. Add 0.5 μl of T4 DNA polymerase (3 U/μl, NEB) to the mixture and incubate at room temperature for 2.5 min. Then, put the reaction mixture on ice immediately and incubate for 10 min. At the same time, thaw chemically competent cells DH5α (200 μl) on ice for ~10 min.
- c. Gently mix the cells with 10 μl of the reaction mixture and incubate the cells on ice for 30 min. Heat shock the cells at 42 °C for 90 s. Incubate the cells on ice for 2 min. Add 800 μl of LB medium to the cells and transfer the cells to 15 ml glass tube. Incubate the cells at 37 °C for 1 h. Then plate the cells on agar plates containing ampicillin antibiotics. Normally, we spread 200 μl of culture onto an agar plate. Incubate the plates upside down at 37 °C for 16 h.
- d. Pick six colonies and inoculate each into LB medium supplemented with ampicillin in 15 ml glass tubes. Grow overnight in a 37 °C rotating incubator. Extract plasmid DNA from the above cultures using the Omega Plasmid Mini kit as per the manufacturer's protocol.
- e. Sequence clones with PA-F and PA-R sequencing primers to confirm the proper insertion of linker-AP tag-duplicated packaging sequence into the pHW2000-PA backbone.
- f. The resulting plasmid is herein referred to as pHW2000-PA-AP tag.

B. Rescue of infectious recombinant rPR8-PA-AP

1. Transfect the cells with rPR8-PA-AP rescue plasmids.
 - a. Transfect 293T cells in Opti-MEM as per the manufacturer's protocol for 6-well plate, and the following per well:
 - 2 × 10⁶ 293T cells
 - 1 μg pHW2000-PB2
 - 1 μg pHW2000-PB1
 - 1 μg pHW2000-HA
 - 1 μg pHW2000-NP
 - 1 μg pHW2000-NA

- 1 µg pHW2000-M
1 µg pHW2000-NS
1 µg pHW2000-PA-AP tag
16 µl Lipofectamine 2000
1.5 ml Opti-MEM
- b. Incubate the transfected cells for 6 h at 37 °C, 5% CO₂. Carefully aspirate the Opti-MEM/lipofectamine mix from the transfected cells, and replace with 1.5 ml of fresh Opti-MEM. After 48 h, transfer the supernatant from the transfected cells into a 2.0 ml microcentrifuge tube. Centrifuge the supernatant for 1 min, 19,419 x g to remove cell debris.
 2. Rescue rPR8-PA-AP from transfection supernatants
- Infection of 10-day-old chicken embryonated eggs**
- a. Candle the 10-day-old eggs using a light egg candler in a darkroom to mark the interface between the air sac and the allantoic cavity. Make a hole with a 10 ml syringe needle in the eggshell. Inoculate each egg with 200 µl of the tissue culture supernatants using a 1 ml syringe. Cover the hole in the eggshell with melted wax, then incubate the infected eggs at 37 °C for 3 days.
 - b. Prior to harvesting the allantoic fluid, incubate the chicken eggs overnight at 4 °C to kill the embryo and coagulate the blood.
 - c. Clean the eggshells with 70% ethanol and open the embryo over the air cavity by tapping with a spoon. Remove the broken eggshell and allantoic membrane with forceps. Stabilize the chicken embryo and collect as much allantoic fluid as possible into a 15 ml centrifuge tube on ice by a 1 ml pipette. Centrifuge for 5 min, 19,419 x g at 4 °C and transfer the allantoic fluid to fresh 15 ml centrifuge tubes. Store the allantoic fluid at 4 °C until they are checked for the presence of rescued virus with a hemagglutination (HA) assay.

Note: For more information about this process, please watch the jove video (<https://www.jove.com/video/2057/generation-of-recombinant-influenza-virus-from-plasmid-dna>). Chicken eggs are SPF-level eggs. Infected-eggs are discarded in autoclavable bags and autoclaved following standard procedures. (Martínez-Sobrido and Adolfo García-Sastre, 2010)

HA assays

- a. HA assays are carried out in V-bottom 96-well plates. Add 50 µl of 1x PBS into each well of the V-bottom 96-well plate.
- b. Add 50 µl of allantoic fluid to the first well and make 2-fold serial dilutions for the following wells. Discard the extra 50 µl from the last well.
- c. Add 50 µl of 1.0% chicken red blood cells (prepared in 1x PBS) to each well.
- d. Incubate the V-bottom 96-well plate for 30 min (until a red dot is visible in the bottom) at RT. A positive viral rescue can be confirmed by HA assay (Figure 3).

Note: For more information about haemagglutination inhibition testing, please see Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza, WHO Part 2.E.

Plaque purification of rescued rPR8-PA-AP

- a. Seed 2×10^5 MDCK cells/well to 90% confluence in 12-well plates using 1.5 ml growth medium per well.
- b. Dilute HA positive allantoic fluid and perform eleven 1:10 dilutions using infection medium.
- c. Heat 1.2% agar until dissolved and place it in a 56 °C water bath. Add TPCK-trypsin to the 2x plaque assay medium at concentration of 2 µg/ml and place it in a 37 °C water bath.
- d. Aspirate the growth medium from the MDCK cells, and wash thrice in 1x PBS.
- e. Add the diluted samples (200 µl) to each 12-well plate, then incubate the well plate for 1 h at 37 °C, 5% CO₂ and shake the plate every 15 min.
- f. Carefully aspirate the inoculums from the MDCK cells, and wash the cells once in 1x PBS.
- g. Mix 2x plaque assay medium plus TPCK-trypsin (7 ml) with 1.2% agar (7 ml) with a ratio of 1:1 and immediately overlay each well with 1 ml mixture, and solidify at room temperature for 30 min.
- h. Invert the plate and incubate for 48-72 h at 37 °C, 5% CO₂ until the plaques are visible (Figure 4A).
- i. Outline the individual plaques and pick about 10 plaques. Using a 200 µl pipet tip, stab the plaque and scrape the cells forming the plaque. Inoculate each plaque into 500 µl of infection medium.
- j. Detect and sequence PA-AP gene from the plaques by RT-PCR using the OMEGA Viral RNA kit and TaKaRa one step SYBR PrimeScript PLUS RT-PCR Kit with PA-F and PA-R primers following the manufacturer's protocol. The correct clonal stocks are amplified in chicken eggs for further use.

Note: For more information about plaque assay protocol, please see Immunoplaque Assay (Influenza Virus), Bio-protocol (Tse et al., 2013).

C. Production of IAV-QDs

1. Seed 5×10^6 MDCK cells/well to reach 80% confluence in 100 mm dishes using 10 ml growth medium per dish.
2. Transfect MDCK cells in Opti-MEM as per the manufacturer's protocol.
15 µg pcDNA3.1(+)-BirA
37.5 µl Lipofectamine 2000
6 ml Opti-MEM containing 50 µM biotin
3. Incubate the transfected cells for 6 h at 37 °C, 5% CO₂. Aspirate the Opti MEM/ lipofectamine mix from the transfected cells and wash twice in 1x PBS.
4. Incubate with rPR8 PA-AP viruses at a multiplicity of infection (MOI) of 10 about 1 ml for 1 h at 37 °C, 5% CO₂. Rock the dishes every 15 min to keep the inoculums evenly distributed.
5. Remove the inoculums from the MDCK cells, wash twice in 1x PBS and add 10 ml infection medium containing 50 µM biotin.
6. After a 2-h incubation, deliver SA-QD625 into MDCK cells by liposomal transfection:

4 µl SA-QD625

10 µl Lipofectamine 2000

1 ml Opti-MEM

7. Harvest cell culture supernatants every 12 h after virus infection and supplement with fresh infection medium containing 50 µM biotin until 3 days post-infection or ~80% CPE.

Note: The purpose of adding biotin during the 6 h incubation after the transfection of the BirA is to internalize biotin into cells before PA protein expression. Biotinylation of PA protein is dependent on BirA catalyzing the ligation of biotin to AP tag. Quantum dots introduced into cells are coupled with biotinylated PA proteins during virus infection.

8. Centrifuge the collected supernatants at 4 °C, 19,419 x g for 10 min and filter through a 0.45 µm filter to remove cell debris.
9. Centrifuge at 4 °C, 153,125 x g for 2.5 h with Beckman SW32 rotor on 5 ml of a 30% (w/v) sucrose cushion in PBS to concentrate the supernatants. Then layer the pellet onto a 20-60% sucrose density gradient and centrifuge at 4 °C, 245,853 x g for 3.5 h with SW41 rotor.
10. Collect fractions containing QD625 under UV excitation (Figure 5), and centrifuge at 4 °C, 161,560 x g for 1.5 h to remove sucrose.
11. Resuspend purified virus in PBS (200-500 µl) to acquire quantum dots-containing influenza virus particles (IAV-QDs).

Note: For more information about concentration of virus by ultracentrifugation, please see Influenza Virus Methods in Molecular Biology part 5: Purification and Proteomics of Influenza Virions (Hutchinson et al., 2018).

D. Transmission electron microscopy of IAV-QDs

1. Load IAV-QDs sample onto carbon-coated copper grids, adsorb for 5 to 10 min, and remove the excess liquid using filter papers.
2. Wash the grids once with PBS and negatively stain with 10 µl of 1% phosphotungstate (PTA) for 20 s at room temperature.
3. Remove the excess phosphotungstate using filter papers and let the copper grids dry naturally.
4. Examine the prepared copper grids under a transmission electron microscope.

Note: For more information about transmission electron microscopy for virus detection, please see Application of transmission electron microscopy to the clinical study of viral and bacterial infections: Present and future (<https://doi: 10.1016/j.micron.2005.10.001>) (Curry et al., 2006).

Data analysis

1. For virus rescue, read the results as indicated in Figure 3 Virus rescue causes hemagglutination of RBC, while no rescue of virus causes the formation of a red pellet of RBC in the bottom of the well.

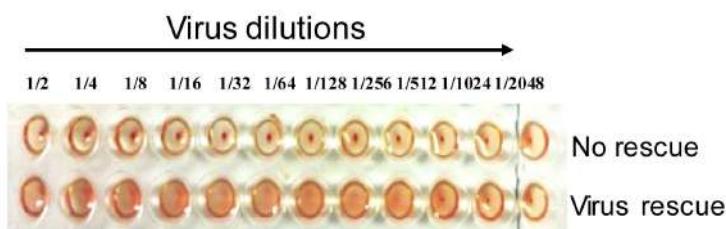


Figure 3. Hemagglutinin assay. A representative result from an HA assay is shown with no detectable levels of virus (top) or presence of virus (bottom).

2. For plaque purification, we picked 10 plaques, as shown in Figure 4A, 5 small plaques (white arrow) and 5 large plaque (black arrow). A single band of the expected size was detected from the purified rPR8-PA-AP by RT-PCR, which is larger than the wild-type PA gene due to insertion of the foreign sequence (Figure 4B).

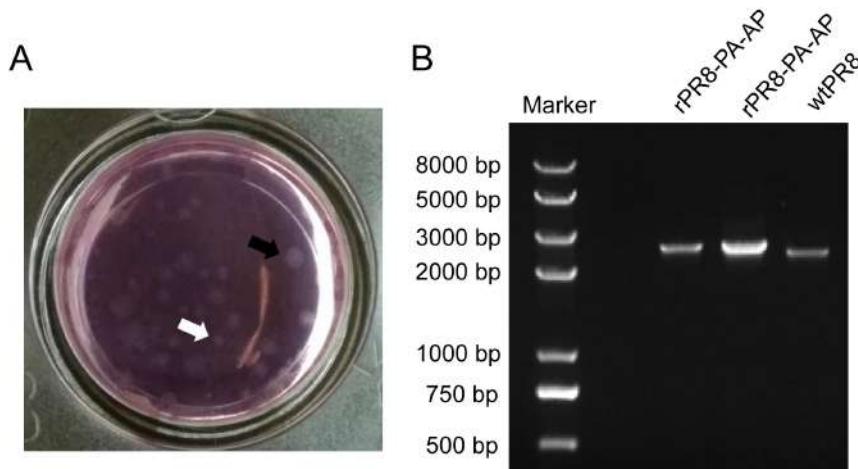


Figure 4. Virus purification. A. Viral plaques appear as white round spots. White arrow indicates small plaque, black arrow indicates large plaque. B. RT-PCR detection of PA-AP gene of purified rPR8-PA-AP.

3. For IAV-QDs production, after sucrose density gradient centrifugation, the labeled virus band shows red color under UV excitation and is located in the 50% sucrose layer, as shown in Figure 5.

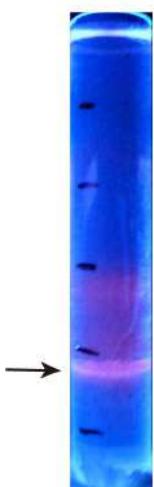


Figure 5. Sucrose density gradient ultracentrifugation. Arrow indicates IAV-QD625 band under UV excitation.

4. For TEM imaging of IAV-QDs, we tested different times and concentration gradients to determine the optimal conditions for IAV-QDs staining. We set staining times from 5 s to 3 min and PTA concentration from 0.25% to 2%. Under the condition of staining with 1% PTA for 20 s, we get the highest-resolution images, as shown in Figure 6. TEM images show the structure of virus as well as dark quantum dots clearly.

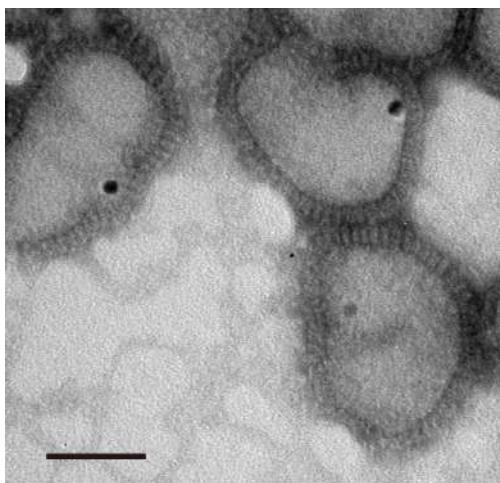


Figure 6. TEM images of IAV-QD625. The dark round dots indicate QD625s, Scale bar: 50 nm.

Recipes

1. LB medium with 100 µg/ml ampicillin
10 g NaCl
10 g Tryptone
5 g yeast extract

1 L ddH₂O

Autoclave at 121 °C, 15 min and let cool to RT

1 ml of 100 mg/ml ampicillin

2. LB agar plate with 100 µg/ml ampicillin

2 g NaCl

2 g Tryptone

1 g yeast extract

3 g agar

200 ml ddH₂O

Autoclave at 121 °C, 15 min and let cool to 55 °C

200 µl of 100 mg/ml ampicillin

3. Cell culture medium

445 ml DMEM

50 ml FBS

5 ml penicillin/streptomycin

4. Infection medium

429 ml MEM

66 ml 7.5% BSA

5 ml penicillin/streptomycin

0.5 µg/ml TPCK-trypsin (final concentration)

5. 0.5% Chicken red blood cell (RBC)

0.5 ml RBC

100 ml physiological saline solution

6. 2x plaque medium

100 ml 2x DMEM

1 µg/ml TPCK-trypsin

7. 1.6% agar

1.6 g low gelling temperature agarose

100 ml ddH₂O

Autoclave at 121 °C, 15 min

8. Sucrose solution

20% (w/v) sucrose: 20 g sucrose, 100 ml 1x PBS

30% (w/v) sucrose: 30 g sucrose, 100 ml 1x PBS

40% (w/v) sucrose: 40 g sucrose, 100 ml 1x PBS

50% (w/v) sucrose: 50 g sucrose, 100 ml 1x PBS

60% (w/v) sucrose: 60 g sucrose, 100 ml 1x PBS

Acknowledgments

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Competing interests

The authors declare that they do not have any conflicts of interests or competing interests.

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Quantification of HIV-2 DNA in Whole Blood

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[Abstract] Time to AIDS infection is longer with HIV-2, compared to HIV-1, but without antiretroviral therapy both infections will cause AIDS-related mortality. In HIV-2 infection, monitoring of antiretroviral treatment (ART) efficacy is challenging since a large proportion of HIV-2-infected individuals displays low or undetectable plasma RNA levels. Hence, quantification of cellular DNA load may constitute an alternative method for monitoring ART efficacy. Moreover, sensitive HIV-2 DNA quantification protocols are also important for the characterization of the HIV-2 reservoirs, and ultimately for the development of HIV-2 cure strategies. We have developed a sensitive and robust HIV-2 DNA quantification protocol based on whole blood as DNA source, including normalization of leukocyte cell numbers using parallel quantification of the single copy porphobilinogen deaminase gene. The specificity and sensitivity of the assay was 100%. The limit of detection was 1 copy and limit of quantification was 5 copies. When applying this protocol to HIV-2 infected, it was found that HIV-2 viral DNA was detectable in individuals in whom viral RNA was undetectable or under quantification level. Thus, this method provides a sensitive approach to HIV-2 DNA viral quantification from whole blood of HIV-2 infected patients.

Keywords: HIV-2, DNA load, Quantification, qPCR, Whole Blood

[Background] Acquired immunodeficiency syndrome (AIDS) can be caused by two viruses, either human immunodeficiency virus (HIV) type 1 (HIV-1) or type 2 (HIV-2) (Azevedo-Pereira and Santos-Costa, 2016). Although these two retroviruses have similar life cycles, they differ in that HIV-2 displays lower pathogenicity, translating into longer progression time to AIDS and reduced transmissibility (Marvin et al., 2015; Azevedo-Pereira and Santos-Costa, 2016). Another distinct feature of HIV-2 is that the plasma viral RNA load of infected individuals is significantly lower, compared with those infected with HIV-1 (Berry et al., 1998; Popper et al., 1999; Andersson et al., 2000). The cellular viral DNA load in peripheral blood has, instead, been suggested to be similar between HIV-1 and HIV-2 infections (Ariyoshi et al., 1996; Popper et al., 2000; Damond et al., 2001). However, others have indicated that also the DNA load is lower in HIV-2, compared with HIV-1, infection (Gueudin et al., 2008). Overall, HIV-2 DNA load quantification studies are relatively few, and most studies have not considered a protocol for a house-keeping gene to quantify the number of cells used in the reaction (Ariyoshi et al., 1996; Gomes et al., 1999; Popper et al., 2000; Damond et al., 2001; Soares et al., 2006; Gueudin et al., 2008; Bertine et al., 2017).

Despite the fact that the estimated time to AIDS development in HIV-2 infected individuals is longer than for individuals with HIV-1, it is clear that HIV-2 will cause AIDS-related mortality without antiretroviral therapy (ART) in infected individuals with long follow-up (Esbjornsson et al., 2018). The monitoring of treatment efficacy is, however, challenging since a large proportion of ART-naïve HIV-2 infected individuals display low or undetectable plasma RNA levels (Berry et al., 1998; Popper et al., 1999; Andersson et al., 2000; Buggert et al., 2016; Honge et al., 2018). Hence, quantification of cellular DNA load may constitute an alternative method for monitoring of ART efficacy.

For simplicity, robustness and the requirement of small volumes of blood, viral DNA quantification protocols based on whole blood, as the source of DNA, are desirable. Currently there are, however, a limited number of publications where HIV-2 DNA quantification has been based on whole blood (Bertine et al., 2017), and the need for protocols combining sensitivity with robustness is still important.

In a recent study (Esbjornsson et al., 2018), we showed that both HIV-1-infected and HIV-2-infected individuals have a high probability of developing AIDS without antiretroviral treatment.

In HIV-1 infection it has been shown that viral DNA load is an independent marker of disease progression and is well correlated with the number of latently HIV-1 infected cells, that comprises the viral reservoir (Parisi et al., 2012; Rouzioux and Avettand-Fenoel, 2018). Comprehensive studies on the HIV-2 reservoirs are, however, lacking. Thus, sensitive HIV-2 DNA quantifications protocols are also important for the characterization of the HIV-2 reservoirs, and ultimately for the development of HIV-2 cure strategies. We therefore decided to develop a sensitive and robust cellular HIV-2 DNA quantification method based on whole blood as DNA source, including normalization of leukocyte numbers using parallel quantification of the single copy porphobilinogen deaminase gene (PBDG) (Raich et al., 1986; Mbisa et al., 2009).

Materials and Reagents

A. DNA extraction

1. Filter tips (1,000 µl filter tip: Sarstedt, catalog number: 70.762.211; 200 µl filter tip: Sarstedt, catalog number: 70.760.211; 20 µl filter tip: Sarstedt, catalog number: 70.760.213)
2. Low Binding Eppendorf tubes (DNA LoBind Snap Cap PCR Tube, 1.5 ml) (Eppendorf, catalog number: 022431021)
3. PM1 cells (Lusso et al., 1995)
4. Whole Blood specimen of HIV-2 infected individuals, storage in -70 °C freezer. Collect samples in EDTA tubes (BD Life Sciences, Vacutainer spray-coated K2EDTA tubes, catalog number: 367841)
5. Whole Blood specimen of uninfected individuals, storage in -70 °C freezer. Collect samples in EDTA tubes (BD Life Sciences, Vacutainer spray-coated K2EDTA tubes, catalog number: 67841)
6. Carrier RNA (poly A) (1350 µg) (Qiagen, catalog number: 1017647) store at room temperature before use, then store at -20 °C
7. Gibco™ RPMI 1640 Medium (Thermo Fisher Scientific, Fisher Scientific, catalog number:

- 11875093), store at 4 °C before opening the bottle
8. Gibco™ Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Fisher Scientific, catalog number: 26140079), storage in -20 °C freezer before heat inactivation, then portion it and store at -20 °C
 9. Gibco™ Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Fisher Scientific, catalog number: 15140122), store at -20 °C
 10. QIAamp DNA Mini Kit (Qiagen, catalog number: 51304), store at room temperature
 11. QIAamp Blood DNA Mini Kit (Qiagen, catalog number: 51104), store at room temperature
 12. Gibco™ Phosphate buffered saline (PBS, pH = 7.4) (Thermo Scientific, Fisher Scientific catalog number: 10010023), store at room temperature
 13. Complete RPMI-1640 medium (see Recipes)

B. Preparation of Standards

1. Filter tips (1,000 µl filter tip: Sarstedt, catalog number: 70.762.211; 200 µl filter tip: Sarstedt, catalog number: 70.760.211; 20 µl filter tip: Sarstedt, catalog number: 70.760.213)
2. Pipette Tips (1,000 µl filter tips: Sarstedt, catalog number: 70.762.100; 200 µl filter tips: Sarstedt, catalog number: 70.760.502; 10 µl tips: Sarstedt, catalog number: 70.1130)
3. Low Binding Eppendorf tubes (DNA LoBind Snap Cap PCR Tube, 1.5 ml) (Eppendorf, catalog number: 022431021)
4. MicroAmp™ Optical 8-Tube Strip, 0.2 ml (Thermo Scientific, Applied Biosystems™, catalog number: 4316567)
5. MicroAmp™ Optical 8-Cap Strips (Thermo Scientific, Applied Biosystems™, catalog number: 4323032)
6. HIV-2 (NIH-Z Strain) Purified Virus and electron microscopy counted (Advanced Biotechnologies Inc, catalog number: 10-127-000), storage in -70 °C freezer
7. Qiagen miRNeasy micro Kit (Qiagen, catalog number: 217084), store at 4 °C
8. DNA oligonucleotides primers were obtained from Invitrogen and listed in Table 1, store oligonucleotides at -20 °C
9. SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific, Invitrogen, catalog number: 12574018), storage in -20 °C freezer. SuperScript™ III One-Step RT-PCR System is supplied with the following:
 - a. SuperScript® III RT/Platinum® Taq Mix (50 µl)
 - b. 2x Reaction Mix (containing 0.4 mM of each dNTP, 3.2 mM MgSO₄) (1 ml)
 - c. 5 mM Magnesium Sulfate (500 µl)
10. Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Invitrogen, catalog number: 11304011), storage in -20 °C freezer. Platinum® Taq DNA Polymerase High Fidelity (20 µl, at 5 U/µl) is supplied with the following:
 - a. 10x High Fidelity Buffer [600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄] (1.25 ml)
 - b. 50 mM MgSO₄ (1 ml)
11. Agarose (Thermo Fisher Scientific, Invitrogen, catalog number: 16500500), store at room

temperature

12. Milli-Q quality water (RNase, DNase free water [dH_2O]) (VWR, catalog number: SH30538.02), store at room temperature
13. AccuGENETM 50x TAE Buffer (Lonza, catalog number: 51216), store at room temperature
14. Gel Red (VWR International, catalog number: 41003), store at room temperature
15. QIAquick PCR purification Kit (Qiagen, catalog number: 28104), store at room temperature

Table 1. Primers used for HIV and PBDG standard preparation

HIV primers used for standard preparation		HIV-2 reference strain BEN accession (GenBank number M30502)		
Name	Sequence 5'-3'	bp	Start	End
BEN_740F	GTG TTC CCA TCT CTC CTA GTC	20	740	760
BEN_791F	AAC AAG ACC CTG GTC TGT TAG	20	791	811
BEN_1640R	GAT TTC AGG CAC TCT CAG AAG	20	1620	1640

PBDG primers used for standard preparation		Relative position of PBDG gene region (GenBank accession number M95623)		
Name	Sequence 5'-3'	bp	Start	End
PBDG-CF1	TGC TCC CAG TTC TGA AGG TGC T	22	4891	4913
PBDG-CR1	AGG CTC CAC CAC TGA AGT AGA G	22	5645	5666
PBDG-CR2	ACT GCC CTA GGC TCC ACC ACT G	22	5638	5659

C. qPCR for HIV-2 DNA detection

1. Pipette tips (1,000 μl filter tips: Sarstedt, catalog number: 70.762.100; 200 μl filter tips: Sarstedt, catalog number: 70.760.502)
2. 96-well qPCR plates (Applied Biosystems™, catalog number: 4346906)
3. Optical Adhesive Film (Applied Biosystems™, catalog number: 4360954)
4. HyClone™ Water, Molecular Biology Grade (RNase, DNase, proteinase free) (Thermo Fisher Scientific, Fisher Scientific, GE Healthcare HyClone SH30538.FS, catalog number: 10247783), store at room temperature
5. HIV-1 infected U937 Cells (U1) (Folks et al., 1987)
6. Maxima probe qPCR Master Mix (2x) (Thermo Scientific, catalog number: K0231), storage in -20 °C freezer. qPCR Master Mix contains the following:
 - a. 2x Maxima Probe/ROX qPCR Master Mix containing Maxima Hot Start Taq DNA

- Polymerase and dNTPs (1.25 ml)
- b. Nuclease-Free Water (1.25 ml)
- 7. Primer and probe sequences for HIV-2 was obtained from Damond *et al.* (2002) (PMID: 12354861). Storage in -20 °C freezer
- 8. Primer and probe sequences of the housekeeping human gene PBGD was obtained from Mbisa *et al.* (2009) (PMID 19020818). Storage in -20 °C freezer
- 9. Agarose (Thermo Fisher Scientific, Invitrogen, catalog number: 16500500), store at room temperature
- 10. Milli-Q quality water (RNase, DNase free water [dH₂O]) (VWR, catalog number: SH30538.02), store at room temperature
- 11. AccuGENETM 50x TAE Buffer (Lonza, catalog number: 51216), store at room temperature
- 12. Gel Red (VWR International, catalog number: 41003), store at room temperature
- 13. 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, Invitrogen, catalog number: 10787018), storage in -20 °C freezer

Equipment

- 1. EVE™ Automated Cell Counter, NanoEnTek (VWR, Avantor, catalog number: 10027-452)
- 2. EVE™ Cell Counting Slides with Trypan Blue (VWR, Avantor, catalog number: 10027-446)
- 3. Eppendorf Microcentrifuge, model: 5415D, with rotor F 45-24-11, AC/DC input 230 V AC, 50-60 Hz (Sigma-Aldrich, catalog number: Z604062)
- 4. Life Eco Thermal Cycler, 96 well gradient, 100-240V, 50-60Hz, 600W, (Bioer Technology Co., catalog number: BTC42096)
- 5. Gilson single-channel/adjustable-volume pipettes: 0.1-2 µl, 2-20 µl, 20-200 µl, 100-1,000 µl (Eppendorf, catalog number: F167380)
- 6. Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Scientific, Applied Biosystems™, catalog number: 4376600)
- 7. NanoDrop ND-1000 spectrophotometer (Thermo Scientific, catalog number: ND-2000C)
- 8. Mini ReadySub-Cell™ electrophoresis system (Bio-Rad Laboratories, catalog number: 1704467)
- 9. UV-Transparent gel tray (Bio-Rad Laboratories, catalog number: 1704435)
- 10. 8-well Comb (Bio-Rad Laboratories, catalog number: 1704463)
- 11. Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratories, catalog number: 1708195)

Software

- 1. Image Analysis Software, Image Lab™ Software (Life Sciences, Bio-Rad)
- 2. Excel 2016 (Microsoft)
- 3. GraphPad Prism 7.0.

Procedure

Main steps of preparation of HIV-2 qPCR:

1. Extract DNA from PM1 cells as outlined below Steps A1 and A2.
2. Prepare PBDG, cellular controls as indicated in Procedure B “Preparation of PBDG standard” by using extracted DNA of PM1 cells.
3. Extract viral RNA -from HIV-2 isolate- to prepare HIV-2 standards, as mentioned in Procedure B “Preparation of HIV-2 standard”.
4. Extract viral DNA from whole blood as indicated in Step A3.
5. Set up HIV-2 qPCR as outlined below Procedure C.

A. DNA extraction

1. Collect 5×10^6 PM1 cells (Lusso et al., 1995) in 1.5 ml Eppendorf tubes and centrifuge for 5 min at $200 \times g$. Discard supernatant and wash pellets twice in 1 ml of sterile PBS. Store dried pellet at -20°C until DNA extraction.

Notes:

- a. *PM1 cell line is a modified CD4+ T-cell clone, which expresses co-receptors such as CCR5 and CXCR4 (Lusso et al., 1995).*
- b. *Maintain PM1 cells in complete RPMI-1640 (Invitrogen) (see Recipes) until passage number 3, after defreezing. Count cells by using Automated Cell Counter.*
- c. *U1s are subclones of U937 chronically HIV-1 infected cells (Folks et al., 1987). These cells are carrying integrated HIV-1 in their genome, thus extracted DNA can be used as a negative control, and to detect the specificity.*
- d. *For negative control, collect 5×10^6 U1 cells (Folks et al., 1987) as the same way as it is mentioned above for PM1 cells. And count cells by using Automated Cell Counter.*

2. To extract cellular DNA of PM1 cells use Qiagen QIAamp DNA Mini Kit according to the manufacturer’s instructions. A single elution of 50 μl buffer AE is used.

Notes:

- a. *DNA extractions should be performed in a cell biology-grade clean hood equipped with separate air-handling mechanisms or under a DNA-free hood. Add extra centrifugation step before elution to decrease the EtOH contamination: $20,000 \times g$ for 1 min. For elution use prewarmed Buffer AE (56°C) and incubate it for 2 min at room temperature.*
 - b. *For negative control, extract DNA from collected U1 cells (Folks et al., 1987) by using Qiagen QIAamp DNA Mini Kit according to the manufacturer’s instructions, then elute in 50 μl buffer AE.*
3. Extract viral DNA from 200 μl of whole blood in the presence of 5 μg carrier RNA using Qiagen QIAamp Blood DNA Mini Kit according to the manufacturer’s instruction. Load DNA to spin column and elute in 50 μl elution buffer.

Notes:

- a. *DNA extractions should be performed in a biosafety cabinet.*
 - b. *Whole Blood sample should be collected in EDTA tubes and stored at -70 °C until DNA extraction.*
 - c. *To reduce degradation of DNA, avoid freezing/thawing of the whole blood samples.*
 - d. *Extraction should be performed by using low-binding Eppendorf tubes.*
4. Store DNA extracts at -20 °C for up to a few weeks.

B. Preparation of Standards

Preparation of HIV-2 standard

1. Prepare the HIV-2 standard by extracting RNA from 1.44×10^{11} RNA-copies/ml of electron microscopy counted HIV-2 particles using Qiagen miRNeasy micro Kit according to the manufacturer's instructions with minor modifications.

Notes:

- a. *HIV-2 control stock solution has a concentration of 7.2×10^{10} viral particles/ml. As two RNA genomes are packed in each viral particle the final stock contains: 1.44×10^{11} RNA-copies/ml = 1.44×10^8 copies/ μ l (7.2×10^{10} multiply with two). Extract a sample (HIV-2) with 5 μ l from the stock + 95 μ l PBS. Extracted RNA should be eluted in 30 μ l. In this case, the concentration of sample is 2.4×10^7 copies/ μ l ((1.44×10^8 x 5)/30). Use 10-fold dilution series to reach 5,000 copies/ μ l RNA concentration.*
 - b. *By using miRNeasy micro Kit purification gives a better yield and allows elution in smaller volume.*
 - c. *To increase the yield of extracted RNA: increase the amount of qiazol reagent from 700 μ l to 1000 μ l. Double incubation time (10 min instead of 5 min). Use 200 μ l of chloroform instead of 140 μ l during extraction.*
 - d. *To improve the yield of extraction add 5 μ l carrier RNA (1 μ g/ μ l) before and after transferring the aqueous phase.*
 - e. *Wash it twice by using 350 μ l RWT buffer and between washing steps add DNase to decrease the amount of DNA contamination.*
 - f. *Elute RNA in 30 μ l of RNase-free water.*
2. Perform cDNA synthesis directly after RNA extraction by using SuperScript III One-Step RT-PCR System and Platinum Taq DNA polymerase. Prepare cDNA synthesis in 25 μ l reaction volume containing 2x reverse transcription buffer (0.4 mM of each dNTP, 3.2 mM MgSO₄), 1 μ l of eluted total RNA, 100 ng forward and reverse primers, SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase and RNase/DNase-free sterile water up to the final volume (see Table 2).

Table 2. Preparation of cDNA synthesis by reverse transcription and first round nested PCR

Reagents	Volume
2x reaction buffer	12.5 µl
Water	8.5 µl
Forward Primer BEN_740F (100 ng/µl)	1 µl
Reverse Primer BEN_1640R (100 ng/µl)	1 µl
One-Step SuperScript™ III RT/PlatinumTaq High Fidelity Enzyme Mix	1 µl
5,000 copies/µl eluted RNA	1 µl
Final volume	25 µl

3. Quantify the HIV-2 gag fragment of RT-PCR (850 base pairs) using the following program: initial cDNA synthesis for 30 min at 50 °C, followed by amplification: initial denaturation for 2 min at 94 °C, 35 cycles of 15 s at 94 °C, 30 s at 50 °C, 45 s at 68 °C and a final elongation step for 5 min at 68 °C.

Note: By using primer pair BEN_740F and BEN_1640R plus strand of HIV-2 gag can be amplified. The estimated size of the amplified fragment is 850 base pairs.

4. Visualize PCR products on 1% agarose gels stained with Gel Red, run at 110 V for 45 min in 1x TAE buffer.
- Purify PCR Product by using QIAquick PCR purification Kit according to the manufacturer's instruction. Load DNA to spin column and elute in 50 µl sterile water. Quantify the concentration of PCR product by using NanoDrop ND-1000 spectrophotometer and visualize it on 1% agarose gel with the same running conditions (see Figure 1).
 - Purified HIV-2 gag should be stored at -20 °C until needed.

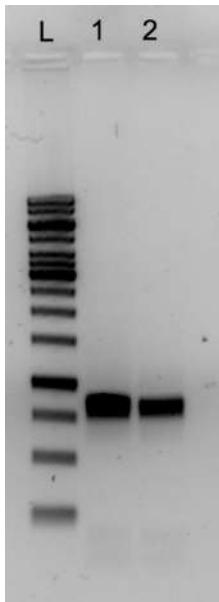


Figure 1. Detection of cleaned PCR product of HIV-2 gag PCR. An example of successful of PCR purification is visualized on 1% Agarose gel stained with Gel red. L = 1 KB DNA ladder, 1 and 2 = HIV-2 PCR products, with the estimated size of 850 bp.

Preparation of PBDG standard

1. Perform host gene standard (PBDG fragment) amplification in two steps (outer followed by nested PCR). Prepare outer PCR in 25 µl solution containing: 10 ng eluted DNA of PM1 cells, Dream Taq DNA polymerase, 5 mM dNTP mix and 100 ng/µl primers (PBDG-CF1 and PBDG-CR2) (see Table 3). Visualize PCR product on 1% agarose gel stained with Gel Red, run at 110 V for 45 min in 1x TAE buffer.
2. Reaction of nested PCR solution should contain: 1 µl of outer PCR product, Dream Taq DNA polymerase, 5 mM dNTP mix and 100 ng/µl primers (PBDG-CF1 and PBDG-CR1). Detect PCR product on 1% agarose gel stained with Gel Red at the same conditions as aforementioned.

Table 3. Host gene performed by 2-step PCR

Reagents	Outer PCR	Nested PCR
	1x	1x
Nuclease Free Water	16.875 µl	16.875 µl
10x Dream Taq Green Buffer	2.5 µl	2.5 µl
dNTP mix (2 mM)	2.5 µl	2.5 µl
Primer Forward (100 ng/µl)	(PBDG-CF) 1 µl	(PBDG-CF1) 1 µl
Primer Reverse (100 ng/µl)	(PBDG-CR2) 1 µl	(PBDG-CR1) 1 µl
Dream Taq Polymerase (5 U/µl)	0.125 µl	0.125 µl
Template	1 µl of PM1 cells (10 ng/µl)	1 µl of outer PCR product
Final volume	25 µl	25 µl

3. Outer and Nested PCR cycling parameters: an initial denaturation 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C and a final elongation step for 5 min at 72 °C.
4. Purify PCR product by using QIAquick PCR purification Kit according to the manufacturer's instruction. Load DNA to spin column, elute in 50 µl sterile water and quantify the concentration of PCR product by using NanoDrop ND-1000 spectrophotometer and detect the product on 1% agarose gel with the same running conditions as mentioned before.

Notes:

- a. *For RNA elution use RNase/ DNase/ proteinase free water.*
- b. *cDNA synthesis and One-Step PCR reactions are conducted in 0.2 ml, MicroAmp™ Optical 8-Tubes.*
- c. *Stock and dilutions must be kept on ice after thawing and mixing, and frozen at -20 °C when not in use. Pipette mix or flick mix samples and dilutions, do not vortex to mix.*
- d. *RNA extractions, dilution of RNA and DNA, PCR setups must be done in the amplicon-free room and should be performed using filtered pipette tips.*
- e. *Modified PCR protocol of Damond et al. (2002) is used for HIV-2 qPCR.*

C. qPCR for HIV-2 DNA detection

Main steps of the qPCR (see Figure 2 for an example of experiment layout): 1) Prepare Real-Time PCR reaction of HIV-2 from extracted DNA of infected and uninfected individuals and with sterile water instead of DNA, as mentioned in Steps C1, C2 and C3. 2) Prepare PCR reaction for HIV-2 standard from HIV-2 gag fragment corresponding to 10⁶ to 1 DNA copies as mentioned in Step C5. 3) Prepare Real-Time PCR reaction of PBDG from extracted DNA of infected and uninfected individuals and with sterile water instead of DNA as mentioned in Step C6. 4) Prepare PCR reaction for PBDG standard from purified housekeeping gene fragment corresponding to 10⁶ to 100 DNA copies as mentioned in Step C7. 5) All reactions need to be performed using a 96-well plate. 6) Carry out qPCR reaction in the conditions mentioned in Step C10. 7) Calculate the concentration of samples by using the formula in Step C11.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard HIV-2 10 ⁶ copies	Infected Individual 1	Infected Individual 9		Standard PBDG 10 ⁶ copies		Infected Individual 1		Infected Individual 9			
B	Standard HIV-2 10 ⁵ copies	Infected Individual 2	Uninfected Individual 1		Standard PBDG 10 ⁵ copies		Infected Individual 2		Uninfected Individual 1			
C	Standard HIV-2 10 ⁴ copies	Infected Individual 3	Uninfected Individual 2		Standard PBDG 10 ⁴ copies		Infected Individual 3		Uninfected Individual 2			
D	Standard HIV-2 10 ³ copies	Infected Individual 4	Uninfected Individual 3		Standard PBDG 10 ³ copies		Infected Individual 4		Uninfected Individual 3			
E	Standard HIV-2 10 ² copies	Infected Individual 5	Uninfected individual 4		Standard PBDG 10 ² copies		Infected Individual 5		Uninfected individual 4			
F	Standard HIV-2 10 copies	Infected Individual 6	Uninfected individual 5				Infected Individual 6		Uninfected individual 5			
G	Standard HIV-2 5 copies	Infected Individual 7	U1 cells' negative control				Infected Individual 7		U1 cells' negative control			
H	Standard HIV-2 1 copy	Infected Individual 8	Negative control of HIV-2 qPCR (sterile water)				Infected Individual 8		Negative control of PBDG qPCR (sterile water)			

Figure 2. Example of the experimental layout. Blue colors indicate the qPCR of HIV-2 gag, while yellow colors stand for Real-Time PCR of PBDG gene. Standard curves are generated from eight (for HIV-2) (10⁶ to 1 copies) and from five dilutions (for PBDG) (10⁶ to 100 copies) dilutions. Each sample and control are prepared in duplicates on a 96-well qPCR plate. DNA of U1 cells stands for negative and specificity controls (as these cells are carrying an integrated HIV-1 genome, but no HIV-2 genome).

1. Prepare the following Real-Time PCR reactions for HIV-2 in 30 µl solution (quantities per sample) containing 15 µl Thermo Fisher Maxima probe qPCR Master Mix, 4 µM of Taqman Probe, 100 ng/µl of each primer and 8 µl of template DNA.

Notes:

- a. Sequences of primers and probe were obtained from Damond et al. (2002) (PMID:12354861).
- b. Extract sample DNA from 200 µl of whole blood as mentioned in Step A3. To improve the yield of extraction use carrier RNA during viral DNA extraction.
- c. Since carrier RNA was used in the extraction, it is not possible to quantify the DNA reliably since spectrophotometric quantification will detect both RNA and DNA in the solution.

2. As a negative control, add 8 µl of sterile water instead of DNA.
3. Set up the same reaction by using DNA from uninfected individuals to ensure that the primers are specific for viral DNA detection.

Note: Extract DNA from 200 µl of whole blood of uninfected individuals in the same way as it was performed for infected samples (see Step A3).

4. Also, prepare the reaction in the presence of 1 µg PM-1 cell's DNA to exclude whether or not human DNA contamination has an effect on the sensitivity of real-time PCR.
5. For quantification, use a series of ten-fold dilutions of the HIV-2 gag fragment corresponding to

10^6 to 1 DNA copies of the standard, and include it in each experiment in order to generate an external standard curve. Each sample and standard should be quantified in duplicate (see Figures 3 and 4).

Note: The most critical step of HIV-2 qPCR is the preparation of standards. Prepare the dilutions by using 1.5 ml low-binding Eppendorf tubes. Mix every tube by pipetting up and down, then centrifuge it before preparing the next dilution.

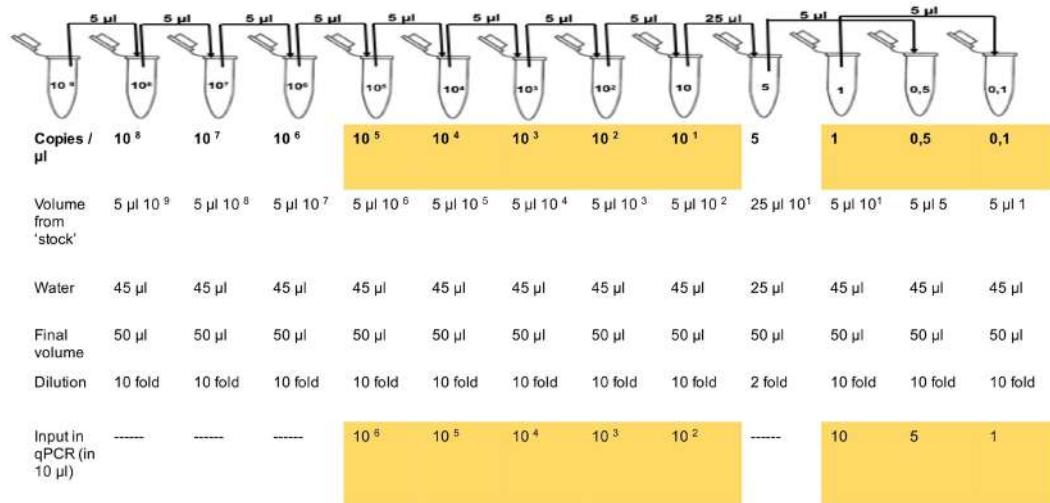


Figure 3. The serial dilution scheme for HIV-2 standard. Prepare serial dilution in 1.5 ml Eppendorf tubes by pipetting 5 μl from 10⁹ 'stock' to 10⁸ copies/ μl tube and add 45 μl water. Pipette up and down to mix and shortly centrifuge it. Using a new tip, pipette 5 μl from tube 10⁸ to tube 10⁷. Pipette up and down to mix and centrifuge it for a few seconds. Repeat the same process for the other tubes. Yellow highlighted shows dilutions used to perform HIV-2 standard curve.

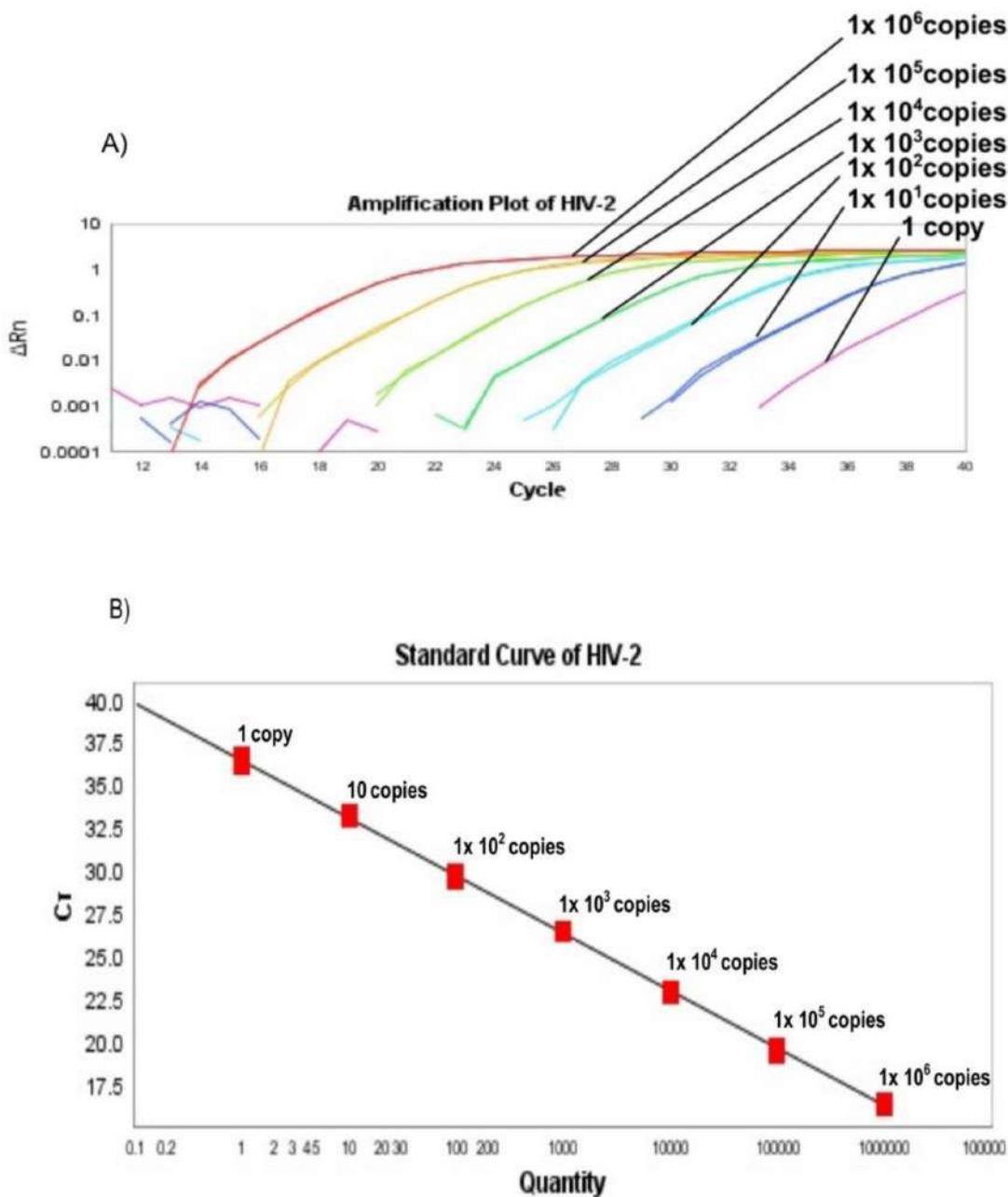


Figure 4. Standard curve of HIV-2 gag fragment. A. Sensitivity test for TaqMan Probe for gene of interest. Amplification plot showed the testing results of the tenfold dilution series of host gene (10^6 , 10^5 , 10^4 , 10^3 , 100, 10 and 1 DNA copies per reaction in duplicate). B. The linear regression analysis between the quantities of host gene (10^6 , 10^5 , 10^4 , 10^3 , 100, 10 and 1 DNA copies per reaction in duplicate) and Ct values. Regression equations were calculated with $y = -3.305 + 38.692$, $R^2 = 0.999$. The red box represents Ct values.

6. Normalize HIV-2 DNA to expression of the housekeeping human gene, *PBGD*. Prepare the following reaction: 12.5 μ l Thermo Fisher Maxima probe qPCR Master Mix, 50 ng/ μ l primers, 2 μ M of probe and 3 μ l of template DNA.

Notes:

- a. Primer and probe sequences of *PBGD* gene were obtained from Mbisa et al. (2009) (PMID 19020818).
 - b. Use extracted DNA of uninfected and infected individuals as a template DNA. For preparation of DNA, see Step A3.
7. For each run, a standard curve should be generated from purified *PBDG* fragment ranging from 10^6 to 100 DNA copies (see Figure 5).

Notes:

- a. Prepare the same reaction for *PBDG* standard as mentioned in Step C6.
 - b. Similar to HIV-2 standard preparation, dilutions of *PBDG* should be performed by using 1.5 ml low-binding tubes. Mixing by pipetting up and down, then centrifugation is necessary before every step of dilution.
8. Distribute the reaction mix in the wells of the 96-well qPCR plate and avoid air bubbles. An example of a plate layout is provided in Figure 2.
9. Cover the qPCR plate with an adhesive plastic film, centrifuge it briefly.
10. Carry out qPCR with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and final extension at 72 °C for 30 s.
11. HIV-2 DNA concentrations should be first reported as HIV-2 DNA copies/ μ l obtained from the qPCR reaction. The numbers of copies of HIV-2 DNA are then normalized to the host gene. The final results are reported as the number of copies/ 10^5 cells. The formula to convert the results is: (HIV-2 DNA copies per μ l)/(PBDG copies per μ l/2 chromosomes per cell) $\times 10^5$ cells = HIV-2 DNA copies per 10^5 cells.

Note: Perform quantitative real-time PCR (qPCR) by using the Applied Biosystems StepOnePlus Real-Time PCR System. Extracted DNA from U1 cells, HIV negative and HIV-1 infected individuals can be used as controls.

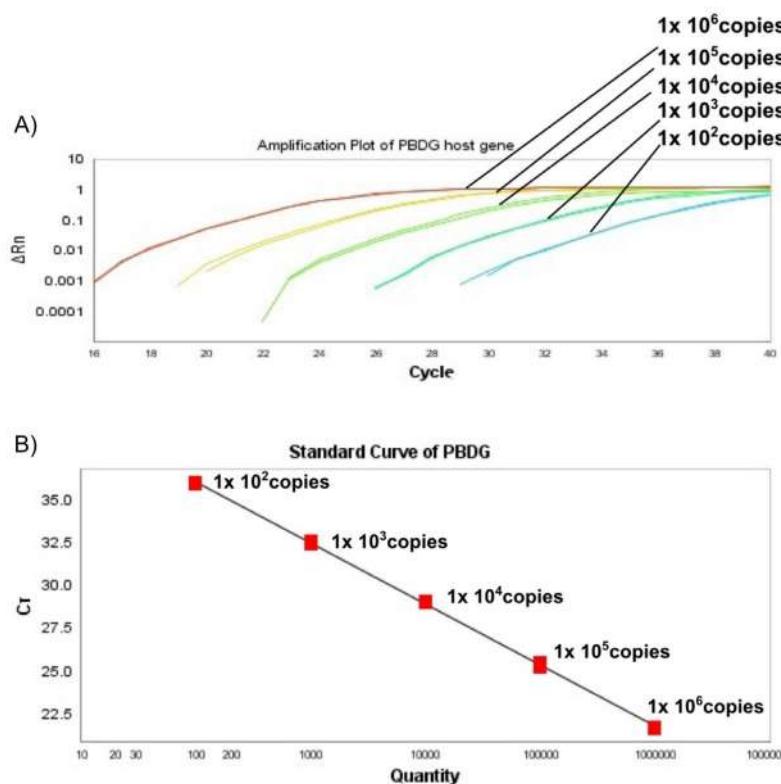


Figure 5. Standard curve for PBDG (host gene). A. Sensitivity test for TaqMan Probe for PBDG host gene. Amplification plot showed the testing results of the tenfold dilution series of host gene (10^6 , 10^5 , 10^4 , 10^3 and 100 DNA copies per reaction in duplicate). B. The linear regression analysis between the quantities of host gene (10^6 , 10^5 , 10^4 , 10^3 and 100 DNA copies per reaction in duplicate) and Ct values. Regression equations were calculated with $y = -3.483 + 39.893$, $R^2 = 0.999$. The red box represents Ct values.

Data analysis

1. First export the obtained copy numbers from AB StepOnePlus Real-Time PCR System. Calculate for each sample the average of copy number of duplicate measurements.
Note: To determine HIV-2 DNA load in whole blood, express HIV-2 DNA copies per cell number using normalization to the porphobilinogen deaminase (PBDG) gene copy number.
2. First present DNA levels as copies per μl obtained in the PCR reaction, after that normalize the level of HIV-2 DNA to the PBDG copies per μl obtained in the PCR reaction. Set up duplicates of samples and standards in each experiment. Use the following formula to convert the results: $(\text{HIV-2 DNA copies per } \mu\text{l}) / (\text{PBDG copies per } \mu\text{l}) / 2 \text{ chromosomes per cell} \times 10^5 \text{ cells} = \text{HIV-2 DNA copies per } 10^5 \text{ cells}$. An example of calculation is provided in Figure 6.

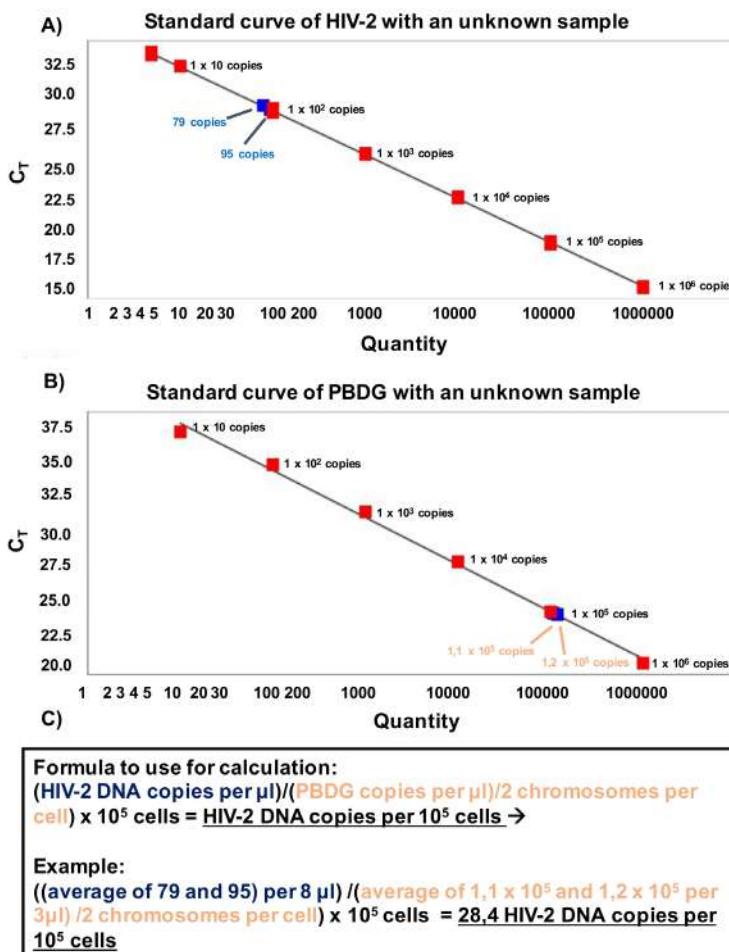


Figure 6. Calculation of HIV-2 DNA copies of an unknown sample. A. Standard curve of HIV-2 *gag* fragment with the copy numbers of unknown samples. The red box represents Ct values of HIV-2 *gag* fragment. Linear regression analysis between the quantities of HIV-2 *gag* gene (10^6 , 10^5 , 10^4 , 10^3 , 100, 10 and 5 DNA copies per reaction in duplicate) and Ct values. The blue box represents HIV-2 *gag* Ct values of unknown samples. B. Standard curve for *PBDG* (housekeeping gene) and unknown sample. The linear regression analysis between the quantities of *PBDG* gene (10^6 , 10^5 , 10^4 , 10^3 , 100 and 1 DNA copies per reaction in duplicate) and Ct values. The red box represents Ct values of *PBDG* and blue box represents Ct values of *PBDG* of unknown sample. C. Formula used to convert the results to HIV-2 DNA copies per 10^5 cells.

- For quantification, the standard with dilutions (see Figure 3) was analyzed in the presence or absence of 1 μg of HIV-2 negative PM1 cell DNA (background DNA). The two curves revealed no differences in their linear range of quantification or PCR efficiency. Moreover, in the presence of 1 μg background DNA, no inhibitory effect was detected (see Figure 7).

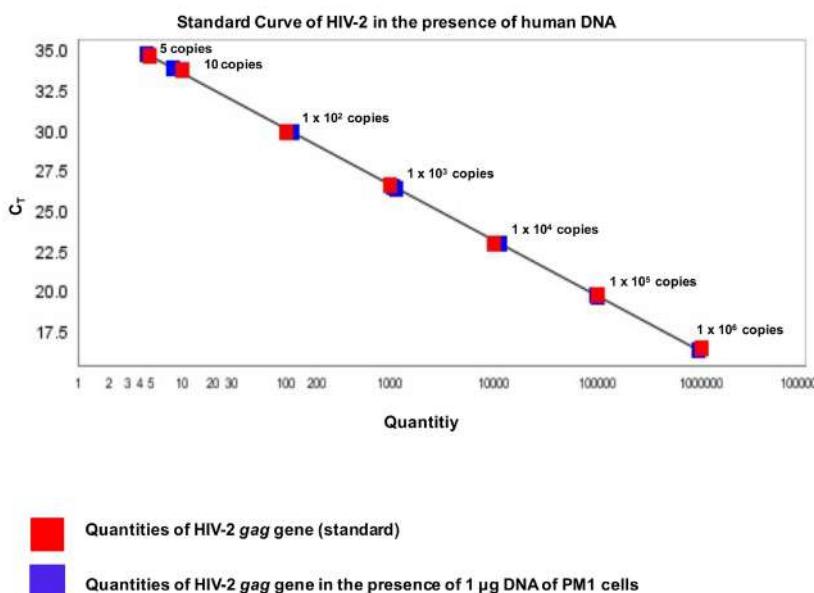


Figure 7. Standard of HIV-2 gag fragment in the presence of 1 μg human DNA. To exclude that background human DNA has an inhibitory effect or not, qPCR was performed in the presence of 1 μg DNA of PM1 cells. The linear regression analysis between the quantities of HIV-2 gag gene (10^6 , 10^5 , 10^4 , 10^3 , 100, 10 and 5 DNA copies per reaction in duplicate) and Ct values in the presence of DNA of PM1 cells. Regression equations were calculated with $y = -3.305 + 38.692$, $R^2 = 0.999$. The red box represents Ct values of standard without human DNA. The blue box represents Ct values in the presence of PM1 cells' DNA.

- Sensitivity of the assay was 100% at five copies/reaction (detected in 14/14 runs, $0.69 \log_{10}$ with SD 0.44), while 78% at one copy/reaction (11/14 runs). Limit of detection was 1 copies/reaction (11/14 runs), the median correlation coefficient was 0.999 (range: 0.998-1.000) and limit of quantification was 5 copies/reaction at CT 33 (SD: 0.9). Median slope was -3.15 (range: -3.00- -3.37) and median efficiency was 97.0% (range: 98.4% to 98.5%).
- LOD and LOQ was calculated by using a formula described by Schwarz et al. (2004). Detection limit of proviral DNA was 0.5 copies/ 10^5 leukocytes.
- To exclude that this method is specific only for HIV-2, run qPCR on extracted DNA of U1 (subclone of U937, which has been chronically infected with HIV-1) cells (Folks et al., 1987), and negative controls, such as extracted DNA from whole blood of HIV negative individuals and extracted DNA from HIV-1 infected patients. As none of the HIV-negative samples, nor HIV-1 infected samples gave positive results, specificity was 100%.

Recipes

- Complete RPMI-1640 medium (pre-warmed at 37 °C before use)
 - RPMI-1640 medium
 - 2.0 mM L-glutamine

10% FBS (Heat inactivate at 56 ± 2 °C for 30 ± 2 min in a water-bath, and portion at 50 ml/centrifuge tubes, and store at -20 °C until use)
1% penicillin/streptomycin solution

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Competing interests

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors have no conflict of interest.

Ethics

The study was approved by the ethical committees of the National Health Ethics Committee in Guinea-Bissau (Ref 038/CNES/INASA/2016) and the Regional Ethical Review Board, Lund University, Sweden (Dnr 2016/426). All participants received information about the study before inclusion and provided oral and written informed consent. To ensure confidentiality, all study data was managed under code.

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Localizing Genome Segments and Protein Products of a Multipartite Virus in Host Plant Cells

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[Abstract] A founding paradigm in virology is that the spatial unit of the viral replication cycle is an individual cell. This concept applied to multipartite viruses—which have a genome composed of two or more nucleic acid segments, each individually encapsulated—implies that all segments constituting a viral genome need to coinfect the same host cell for replication to occur. Would this requirement be verified, it would constitute a major cost for extreme cases of multipartition such as the *Faba bean necrotic stunt virus* (FBNSV, nanovirus) whose genome is composed of eight complementary segments, each encoding a single gene (Grigoras *et al.*, 2009). To address this question, we followed the distribution of the FBNSV genome segments by fluorescence *in situ* hybridization combined to immunolocalization of the replication-controlling viral protein within the cells of the host plant: *Vicia Faba*.

A rapid and efficient protocol to localize viral transcripts in plant and insect hosts has been developed earlier (Ghanim *et al.*, 2009). We here improve this method by using random-primed labeled probes and apply it to the detection and quantification of the individual segments composing the FBNSV genome. Moreover, we combine this technique with immunolocalization so that both viral segments and proteins can be visualized within the same samples.

Keywords: *In situ* hybridization (ISH), Immunolocalization, Plant, Virus, Multipartite virus, FBNSV, Fluorescence microscopy, Nanovirus, *Vicia Faba*

[Background] Fluorescence *in situ* hybridization (FISH) and immunolocalization are essential techniques to localize specific nucleic acids and proteins respectively. These techniques have been successfully used either separately or in combination in a number of studies. Generally, *in situ* hybridization protocols include numerous steps, resulting in a cumbersome and time-consuming technique. The protocol developed by Ghanim *et al.* has considerably simplified this technique (Ghanim *et al.*, 2009). When applied to our pathosystem (FBNSV, *Vicia faba*), however, this protocol yielded poor and/or non-specific signals. Although FBNSV is a circular single-stranded DNA phytovirus like the *Tomato yellow leaf curl virus* (TYLCV) on which Ghanim and coauthors tested their protocol, the FBNSV genome is composed of eight molecules of around 1 kb each, sharing common regions which sometimes correspond to the almost quasi-totality of their non-coding regions (Grigoras *et al.*, 2010). We thus sought to improve this protocol for the detection of individual FBNSV genomic segments by using, instead of fluorescent oligonucleotides, random primed probes generally used for *in situ* localization experiments on chromosomes. We were able to couple it with immunolocalization for visualization of nucleic acid and proteins *i.e.*, covisualization of genetic information and translation products within the same cells.

By combining these two methods, we showed (i) that the FBNSV genomic segments do not colocalize in individual host cells, and (ii) that the viral protein involved in the replication of all segments is often found within cells lacking the segment encoding it; suggesting that viral expression products are able to move away from the cells where they are produced. We believe that our methodology could easily be adapted to locate and quantify (single-stranded) nucleic acids in different tissues with great specificity. For instance, this protocol has also been successfully used to locate the FBNSV segments within gut cells of its aphid vector and TYLCV genome within host plants.

Materials and Reagents

Note: Some of the reagents and materials described below are optional depending on the targeted sequence and tissue (see Procedure below for further details).

1. Microscopy slides (Dutscher, catalog number: 068763)
2. Coverslips (Dutscher, catalog number: 100031)
3. Nylon membrane 0.45 µm pore size (Sigma, catalog number: GERPN203T)
4. Whatman paper
5. 24-well plate (Dutscher, catalog number: 353226)
6. Microcentrifuge tube (Dutscher, catalog number: 033297)
7. Embryo dish 30 mm (Delta microscopies, Ems, catalog number: 70543-30)
8. Wizard SV Gel PCR Clean-Up System (Promega, catalog number: A9282)
9. GoTag DNA polymerase kit (Promega, catalog number: M3001)
10. Paraformaldehyde (Delta microscopies, Ems, catalog number: 15713), store at room temperature
11. PBS buffer 10x pH 7.4 (Dutscher, biosolve, catalog number: 91549), store at room temperature
12. Tween 20 (Merck, Sigma-Aldrich, catalog number: P1379), store at room temperature
13. Glycine (Merck, Sigma-Aldrich, catalog number: G8898), store at room temperature
14. Ethanol (AnalyticLab, Honeywell, catalog number: 32221), store at room temperature
15. Chloroform (Merck, Sigma-Aldrich, catalog number: 32211), store at room temperature
16. Acetic acid (Merck, Sigma-Aldrich, catalog number: 33209), store at room temperature
17. Low melting agarose (Ozyme, Lonza, catalog number: LON50080), store at room temperature
18. RNase (Merck, Sigma-Aldrich, catalog number: R6513), store at -20 °C
19. Tris (Carl Roth, Pufferan, catalog number: AE15.3), store at room temperature
20. NaCl 5 M (Merck, Sigma-Aldrich, catalog number: S3014), store at room temperature
21. Sodium dodecyl sulfate (SDS) 10% (Dutscher, Biosolve, catalog number: 19812323), store at room temperature
22. Deionized Formamide (Eurobio, catalog number: GHYFOR01-01), aliquot and store at -20 °C after opening
23. BioPrime DNA labeling system (Fisher Scientific, Invitrogen, catalog number: 18094-011), store at -20 °C

24. Chromatide Alexa Fluor 488-5-dUTP (Fisher Scientific, Molecular probes, catalog number: 10173952), store at -20 °C
25. Chromatide Alexa Fluor 568-5-dUTP (Fisher Scientific, Molecular probes, catalog number: 10413222), store at -20 °C
26. Herring sperm DNA (Sigma, catalog number: D3159), store at -20 °C
27. Bovine serum albumin (Carl Roth, catalog number: 8076.4), store at 4 °C
28. Goat anti rabbit Alexa Fluor 488 IgG (H+L) (Fisher Scientific, Invitrogen, catalog number: 10082502), store in 50 % glycerol at -20 °C
29. Goat anti rabbit Alexa Fluor 594 IgG Conjugate (Fisher Scientific, Invitrogen, catalog number: 10266352), store in 50 % glycerol at -20 °C
30. Vectashield mounting medium with DAPI (Eurobio, Vector, catalog number: H-1200), store at 4 °C
31. Carnoy 6:3:1 (Recipe 1)
32. RNase solution 100 µg/ml (Recipe 2)
33. dNTP fluo mix for the Bioprime DNA labeling (Recipe 3)
34. Hybridisation buffer (Hb Buffer) (Recipe 4)
35. 1 M Tris-HCl, pH 8 (Recipe 5)
36. 5 M NaCl (Recipe 6)
37. 0.1 M glycine in PBS 1x (Recipe 7)
38. 5% Bovine Serum Albumin (BSA) (Recipe 8)

Equipment

1. Vibratome (MICROM, model HM650V)
2. Confocal microscope (ZEISS, model LSM700)
3. Phosphorimager (Typhoon FLA 9000)
4. UV light (254 nm) (UVilite, UVItec CL-E508.G)
5. 37 °C oven
6. Water bath
7. Rotator

Software

1. Zeiss Efficient Navigation (ZEN) (ZEISS, version 2009)
2. Image J (National Institutes of Health, version 1.50 c, <https://imagej.nih.gov/ij/download.html>)
3. JMP (version 13.2.0, https://www.jmp.com/fr_fr/home.html)

Procedure

A. Samples preparation

1. Samples fixation

- a. Collect samples (see the note* below for the choice of samples) in PBS 1x and add paraformaldehyde (PFA) right after sampling to obtain a solution of PFA at 4% in PBS 1x with 0.2% Tween-20**. We recommend taking at least one sample from at least 10-12 individual plants coming from three different inoculation trials. For comparison purposes, samples should be collected from plants at the same development and infection stages. Incubate under agitation overnight at 4 °C.

Notes:

- i. *Choose the samples to collect carefully by thinking in which parts you are most likely to find virus accumulations. In our case, we choose to work on petioles.
 - ii. **Pay attention to use a volume of fixator high enough to completely cover the fragment collected: use at least 2/3 volume of fixator for 1/3 volume of sample.
- b. On the next day, neutralize PFA with a PBS 1x solution containing 0.1 M glycine (Recipe 7). Incubate for 15 min at room temperature (RT).
Note: The volume of “PBS 1x solution containing 0.1 M glycine” should be adapted to the sample you are working with (petioles, leaves, roots...); samples must always be entirely covered by the liquid.
 - c. Rinse the samples for 5 min with PBS 1x. Replace PBS with a 70% ethanol solution. Samples can be stored in this solution at 4 °C for up to a month.

2. Samples sectioning

Note: Depending on the localization of the nucleic acid of interest, and on the tissue or organ analyzed, this step could be optional.

- a. Embed the fixed samples in 8% low melting agarose (diluted in either distilled water or PBS 1x). Pour the liquid agarose in a 24-well plate, or anything else convenient for the samples, until the agarose completely covers the sample. Place the tissue fragments straight in the middle of the well and let the agarose solidify at 4 °C for at least 4 h (Figure 1).



Figure 1. Embedded samples. *Vicia faba* petioles embedded in 8% low melting agarose in a 24-well plate before sectioning.

- b. Proceed to the sectioning with a vibratome. For instance, the following parameters were used to section *Vicia faba* petioles using the MICROM vibratome:

thickness: 100 µm

frequency: 100 Hertz

program: 50

amplitude: 0.6 mm

method: one cut after another

speed: 23 mm/s

All of the above parameters—including the percentage of agarose—should be adjusted according to the hardness of the sample. For example, in the case of a softer sample, decrease the percentage of agarose. Based on our experience, 8% is around the limit of agarose solubility.

3. Samples discoloration

For plant samples, bleach them by incubating them under gentle agitation at RT in a Carnoy 6:3:1 solution (Recipe 1). As soon as the samples have totally lost their green color (step unnecessary for tissues such as roots explants), stop the reaction by replacing the Carnoy solution with a PBS 1x solution. For instance, bleaching of *Vicia faba* petiole's sections takes an hour (Figure 2).



Figure 2. Effect of Carnoy 6:3:1 discoloration on sectioned samples. *Vicia faba* sectioned petioles before (left) and after (right) discolored using the Carnoy solution.

4. RNase or DNase treatment

Depending on the nature of the genetic material you want to localize, you can proceed to an

RNase or DNase treatment. Since FBNSV is a single-stranded DNA virus and because we were interested in localizing its genomic segments only, we RNase-treated our samples.

- a. Immerge the samples in 500 µl of a 100 µg/ml RNase solution (Recipe 2) for 45 min at 37 °C.
- b. Rinse 1 time for 5 min in PBS 1x.

B. Probes preparation

DNA amplicons (Table 1) corresponding to the coding regions of each genomic segments were amplified by PCR using the GoTaq Polymerase kit (Promega) and the conditions summarized in Table 2. PCR products were then run on a 1% agarose gel and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). These amplicons were then used as templates to produce segment-specific probes. DNA labeling was performed using the Bioprime DNA labeling Kit of Invitrogen. Two modifications were made to the kit protocol based on the recommendations of the Plant molecular cytogenetic platform of Rennes (INRA) and of the Cytogenetic and the imaging center of UMR AGAP, Montpellier CIRAD (Personal communications). First, for direct labeling, Biotine molecules were replaced with Alexa Fluor enabling a direct labeling. Second, the incubation time was increased to allow enhanced labeling.

Below is the kit labeling procedure with the two changes made (in bold font). All of the reagents are included in the kit—except for the dNTP Fluo mix (Recipe 3).

1. Dissolve 25 to 500 ng (we used 200 ng of PCR product) of DNA in 5-20 µl of dilute buffer in a microcentrifuge tube. On ice, add 20 µl of 2.5x Random Primers Solution. Denature by heating for 10 min in a boiling water bath. Immediately cool on ice for 15 min.
2. On ice, add (to the DNA and primers mixture prepared in Step B1):
 - a. **10 µl of dNTP Fluo mix (instead of the 10x Biotine dNTP mixture, see Recipe 3 for preparation).**
 - b. And distilled Water to a total volume of 49 µl.
3. Mix briefly by vortexing or pipetting.
4. Add 1 µl of Klenow Fragment. Mix gently but thoroughly. Centrifuge at maximum speed (no specific recommendation is included in the manufacturer notice, when we proceed to it, we do it at maximum speed of a bench centrifuge) for 15-30 s.
5. **Incubate at 37 °C for at least 12 h.**
6. Add 5 µl of Stop Buffer and mix.

The probes can be stored at -20 °C for months. The probes chosen must be specific of the DNA or RNA sequence you want to localize. The specificity of the probe can be checked following the procedure detailed in the next section.

Table 1. Primers used to prepare segment-specific fluorescent probes (from Sicard *et al.*, 2019)

Name	Primer sequence	Size	Segment
NV C-297 F	ATGGGTCTGAAATATTCCTC	510nt	C
NV C-806 R	TTAATTAAATTACAATCTCC		
M ORF For2	GCTGCGTATCAAGACGCAC	268nt	M
M ORF Rev2	TTCTAGCATCCCAATTCC		
N ORF For	TGGCAGATTGGTTTCTAGT	450nt	N
N ORF Rev	TTCTGAGTGAATGTACAATAAACATT		
R ORF For	ACATTAATAATCCTCTCTCTCCTA	810nt	R
R ORF Rev	CCTATCATCACTAACATGCC		
S ORF For	AAATGGTGAGCAATTGGAA	350nt	S
S ORF Rev	GCCTATGATAGTAATCATATCTTGACA		
U1 ORF For	TTGGTCGATTATTGTTGGTT	455nt	U1
U1 ORF Rev	AATATCTCATTAGCATTAAATTACATTGAA		
U2 ORF For	TTATGGATGCCGGCTT	361nt	U2
U2 ORF Rev	CATGAAGTATTAGAATAACGAACCTGAA		
U4 ORF For	AGCAGGTTATGGAATGTAGG	313nt	U4
U4 ORF Rev	ATAGATTCCCACAATCGCT		
R1 For	ACATTAATAATCCTCTCTCCTA	282nt	
R1 Rev	CTCTAAGCGTACTTGCA		R
R2 For	GAAGAAAGCTATTGCTACTTGG	406nt	
R2 Rev	CCTATCATCACTAACATGCC		
Nmys2-Red	TTACATCATAAGTGCTGAACTATAAGGCACAACTAA	40 nt	N
Nmys7-Green	TTACATCATATTACCAACTGTTGCTATCCCACAACTAA	40 nt	N
Smys1-Red	GTTTTTCATTAACCTCTCACTATAAACTGCCACAGTG	40 nt	S
Smys8-Green	GTTTTTCATTAATATAAGTCATAATTCCCTGCTCCACAGTG	40 nt	S

Table 2. PCR conditions followed to prepare the segment-specific fluorescent probes. Note that these conditions need to be adjusted according to the polymerase used, the primers and the size of the amplicons.

Reagents		Procedure		
Components	Final concentrations	Step	Temperature	Time
GoTaq reaction buffer	1x	Initial Denaturation	95 °C	2 min
GoTaq DNA polymerase	0.5 unit	40 Cycles	95 °C	45 s
dNTP	0.2 mM each		59 °C	45 s
Primers Forward + Reverse	0.5 µM		72 °C	45 s
Template DNA		Final Extension	72 °C	5 min
Nuclease-free water		Hold	4-10 °C	

C. Specificity of the probe

1. First denature samples (mandatory when starting with double-stranded nucleic acid) by incubating them at 95 °C (or in boiling water) for 10 min immediately followed by placing them on ice.
2. Each of the denatured samples is pipetted on a nylon membrane and allowed to dry. The edges and names of each drop are penciled directly on the membrane (Figure 3).
The samples may include DNA extracted from the tissue of interest containing or not the targeted nucleic acid as well as some purified DNA containing (or not) the targeted nucleic acid (e.g., a plasmid containing the gene of interest). For instance, we spotted 1 µg of each of the eight plasmids, each containing one of the eight FBNSV segments, separately on the membrane. 1 µl of DNA extract from FBNSV infected and uninfected plants were also added to the membrane. Each sample should contain at least 45 ng of the target in order to be detected.
3. Fix the DNA onto the membrane using ultraviolet for a few seconds (UV linker, UVelite, UVItec).
4. Pre-hybridize the nylon membranes for 2 h at 37 °C on a rotator using Hb buffer (Recipe 4) containing 1% (w/v) of BSA and 2 mg of denatured herring sperm DNA.
5. Add 10 µl of the denatured probe previously obtained (Procedure B). Denature samples by heating for 10 min at 95 °C (or in boiling water) followed by an immediate cooling on ice for 15 min.
6. Incubate for at least 2 h at 37 °C on a rotator. This incubation step can also be done overnight.
7. Rinse the membranes three times using hybridization buffer, for 5 min each, and once with PBS 1x for 5 min. Do the rinse steps at room temperature.
8. Dry the membranes with Whatman paper.
9. Results can be visualized using a phosphorimager.

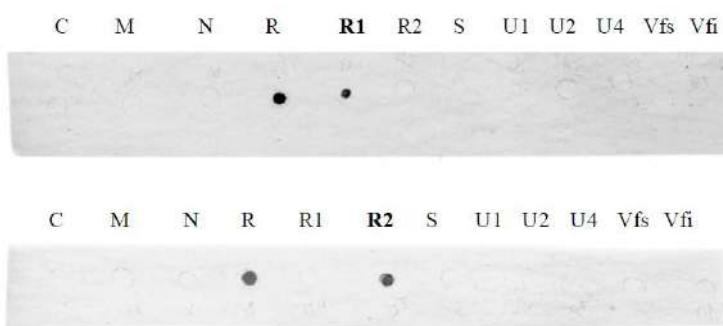


Figure 3. Results obtained using the R1 probe (upper picture) and the R2 probe (lower picture) after revelation with the phosphorimager. 1 µg of each purified plasmid DNA containing one of the eight FBNSV genomic segments (C, M, N, R, S, U1, U2, U4) were spotted on a nylon membrane. 200 ng of PCR purified product corresponding to two different regions of the segment R (R1 and R2) were also spotted on the membrane. Finally, 1 µl of DNA extracted from a healthy plant (Vfs) and 1 µl of DNA extracted from an FBNSV-infected plant (Vfi) were

used. The upper and lower membranes were incubated with the R1 and R2 probes respectively. The results obtained confirm the high specificity of each probe. Note that no signal was obtained here for the FBNSV-infected plant sample when using either of the two R probes due to the very low concentration of the segment R in the amount of plant DNA used.

D. *In situ* hybridization

1. Rinse the samples obtained in Procedure A for 5 min, 3 times with Hybridisation Buffer (Hb buffer, Recipe 4).
2. Dilute 20 µl of labeled DNA probe (with Alexa Fluor 488 or/and Alexa Fluor 568) in Hb buffer in a total volume of 600 µl. Adjust the volume to the amount of material. For example, we used 300 µl of this solution in an embryo dish of 30 mm.
3. Since the labeled probes obtained by Random priming (Procedure B) are double-stranded, denature the solution for 10 min at 95 °C or in boiling water and cool immediately for 15 min on ice. Incubate the samples with the probe solution overnight at 37 °C in a dark place (e.g., incubator). As a general rule, since the fluorochromes are light-sensitive, avoid prolonged exposure to light.
4. Recover the probe solution. In our hands, this solution could be used up to 4 times with no significant decrease of the intensity of the signal. Store the solution at -20 °C in a tube protected from light.
5. Rinse the samples for 5 min 3 times in Hb Buffer (Recipe 4).
6. Rinse 2 times more with PBS 1x for 5 min.

Note: If no other experiment is foreseen, prepare slides for microscopy observations (Procedure F).

E. Immunolocalization

Immunolocalization protocol can immediately follow *in situ* hybridization (Figure 4).

1. Incubate the samples in BSA 5% in PBS 1x solution (Recipe 8) for 1.5 h at room temperature (RT) to block aspecific sites.
2. Split samples into two batches. Incubate one batch with a fresh PBS-BSA 5% solution containing the primary antibody (diluted at the ad hoc concentration). For the second batch, simply replace the PBS-BSA 5 % with a fresh solution (this represents the negative control). Incubate overnight at 4 °C.

Note: Regarding the immunolocalization experiment, you can first try using the primary antibody at a concentration 10x higher than the one used in Western blots. Then, adjust accordingly.

3. Rinse all samples 3 times each for 10 min in PBS 1x + 0.05% Tween-20.
4. Replace the solution by a PBS-BSA 5% solution containing the secondary antibody–diluted at the required concentration (see manufacturer instruction). In our experiments, we used a goat anti-rabbit IgG Conjugate Alexa Fluor 488 at a concentration of 2 µg/ml and a goat anti-rabbit IgG Conjugate Alexa Fluor 594 at 5 µg/ml. Incubate for 1 h at 37 °C.

5. Rinse 3 times each for 10 min in PBS 1x + 0.05% Tween-20.

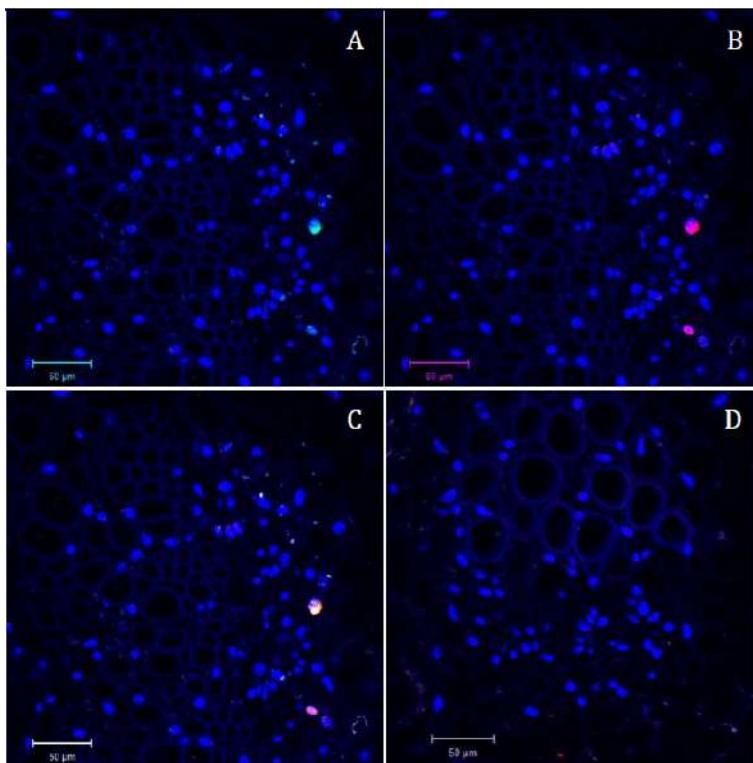


Figure 4. Confocal microscopy pictures of double labeling (FISH/Immuno) experiment obtained following our protocol. Cross sections of infected faba bean petioles stained with DAPI (in blue) and hybridized with the S-A488 probe in green and M-Rep protein antibody in red. A. Green channel (S probe = part of the segment encoding the capsid protein) merged with the blue channel (DAPI). B. Red channel (M-Rep protein localization = immunolabeling of the movement protein) merged with the blue channel (DAPI). C. Merged image of the three channels. D. Merged picture of the three channels of the control sample (petiole of non-infected faba bean plant) taken in the same conditions as Figure 4C; only autofluorescence of the tissue and blue signal of the DAPI nuclei staining could be observed.

F. Preparation of microscopy slides

For microscopy observations, mount samples in Vectashield with DAPI (if interested in localizing cell nuclei) or any other mounting media (e.g., glycerol..., Figure 5).

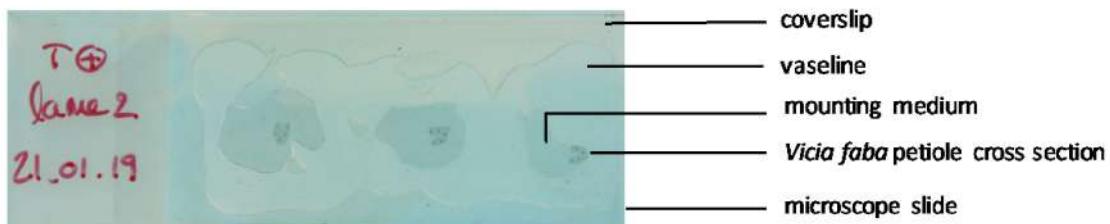


Figure 5. Microscopy slide ready to be observed and mounted as described in the note below. A Vaseline chamber was made prior to adding the mounting medium and the cross section to avoid movement of the preparation during long image captures.

Note: For microscopy slides, depending on the thickness of your samples, you can use Vaseline to make a kind of “incubation chamber” on the slide before adding the mounting media and the coverslip.

G. Microscopy observations

All observations were made using an LSM700 Confocal Microscope (ZEISS) with the ZEN Software. Parameters were adjusted to obtain sufficient resolution and fluorescence intensity signal recovery-in a chosen series of infected plant exhibiting a high intensity of fluorescence-without saturation points. Once these parameters were set, all of the images were acquired using the same parameters so that they could easily be compared with one another.

In our case, images were taken with a 40x water immersion objective with a resolution of at least 512 x 512 and with a pinhole aperture of 1 Airy Unit so as to work in confocal mode.

We set on a configuration with 3 sequential tracks, one for each fluorochrome used.

1. Excitation parameters were as followed:

laser 405 nm for DAPI

laser 488 nm for Alexa Fluor 488

laser 555 nm for Alexa Fluor 568

Fluorescence emission was collected on the photomultiplier 1 and the variable secondary dichroic beam splitter was set so as to recover fluorescence up to:

2. 490 nm for DAPI
3. 535 nm for Alexa 488
4. 615 nm for Alexa 568

Data analysis

Analyses were run using maximum intensity projections so that all the fluorescence emitted in the whole nuclei was accounted for. These microscopic images were analyzed using Image J software.

1. First of all, in order to minimize the signal caused by the autofluorescence of the tissues—either natural or induced by fixation, grey level intensities coming from the green and red tracks were decreased independently on each photo until no green or red fluorescence could be visually detected in tissues where the FBNSV is known to be absent (*i.e.*, in xylem and mesophyll; the FBNSV being a phloem restricted virus). This computer process was done in the exact same way for all pictures and applied equally to the whole surface of the image (Figure 6).

Note: The aim of this step was to simply distinguish infected cells from non-infected ones with a high level of background autofluorescence. As a consequence, only cells in which green or red fluorescence levels exceeded the level of background fluorescence were considered for

further analysis. As we were mainly interested in the cells in which our ssDNA virus can replicate, we focused our analyses on phloem nucleated cells; nuclei stained using DAPI.

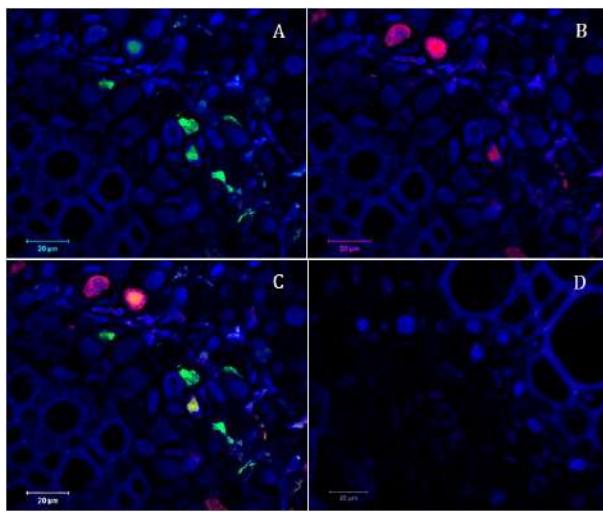


Figure 6. Confocal microscopy pictures obtained following our protocol. Cross sections of infected faba bean petioles stained with DAPI (in blue) and hybridized with the R-A488 probe in green and N-A568 probe in red. A. Green channel (R probe = part of the segment encoding the replication protein) merged with the blue channel (DAPI). B. Red channel (N probe = part of the segment encoding the nuclear shuttle protein) merged with the blue channel (DAPI). C. Merged image of the three channels. D. Merged picture of the three channels of the control sample (petiole of non-infected faba bean plant) taken in the same conditions as picture C; only autofluorescence of the tissue and blue signal of the DAPI nuclei staining could be observed.

2. On the images, we manually delineated the DAPI stained area (corresponding to the nucleus in each cell) using a cursor and quantified intensity of grey level values for each pixel using the ImageJ software. The intensity of grey level was also determined for both green and red fluorescence.
3. We then calculated the average pixel fluorescence intensity value for each nucleus and each fluorochrome. We thus obtained an average intensity value (in arbitrary fluorescence unit) for both green and red fluorescence for each selected individual cell within an infected petiole (the FBNSV replicate and accumulate in the nucleus of infected cells).
4. In order to check the reliability of our analysis, we produced two probes respectively targeting the two moieties of the same segment R: “probe-R1” and “probe-R2”, one labeled green and the other red. In such a control experiment, we could confirm that absolutely all nuclei with a significant red signal also had a significant green signal. When quantifying these green and red fluorescence, we observed a highly significant correlation, logically showing that the accumulation of one part of a genome segment within a cell is clearly correlated to that of the other part of the same segment. Around 50 infected cells were analyzed by condition.
5. Images acquired from the same slide with different resolutions (512 x 512 and 1024 x 1024) and at different times post labeling (2 weeks between the two-time points) were analyzed and led to

the same results.

6. Linear regression analyses were performed with the JMP software.

All details of the data analysis can be found in the original research article (Sicard *et al.*, 2019).

Notes

1. Always use both infected tissues and uninfected tissues as controls.
2. A minimum of 3 repetitions per condition should be run.

Recipes

1. Carnoy 6:3:1

6 ml chloroform

3 ml ethanol

1 ml acetic acid

2. 100 µg/ml RNase solution

Dilute your RNase solution with PBS 1x so as to obtain a 100 µg/ml solution

3. dNTP fluo mix for the Bioprime DNA labeling

Dilute dNTP solutions in H₂O so as to obtain 1 mM solution of dATP, dGTP, dCTP and 0.5 mM of dTTP and dUTP-Alexa

For example, mix:

5 µl of 10 mM dATP

5 µl of 10 mM dGTP

5 µl of 10 mM dCTP

2.5 µl of 10 mM dTTP

25 µl of 1 mM dUTP-Alexa (Alexa 488, 568 or others depending on the microscope equipment)

7.5 µl of H₂O

4. Hb Buffer (Ghanim *et al.*, 2009)

20 mM Tris HCl, pH 8

0.9 M NaCl

0.01% (w/v) SDS

30% (v/v) deionized formamide

pH = 8

For a volume of 100 ml:

2 ml Tris HCl pH8 1 M

18 ml NaCl 5 M

100 µl SDS 10%

30 ml deionized formamide

50 ml H₂O

5. 1 M Tris-HCl, pH 8
121.1 g of Tris base
 - a. Complete with water to 800 ml
 - b. Adjust to pH 8 by adding concentrated HCl (around 42 ml)
 - c. Adjust the volume of the solution to 1 L with H₂O
6. 5 M NaCl
292.2 g NaCl
Complete with water to 1 L
7. 0.1 M glycine in PBS 1x
Dissolve 7.5 g of glycine in PBS 1x to a final volume of 1 L
8. Bovine Serum Albumin (BSA) 5%
Dissolve 5 g of BSA in 10 ml of PBS 1x

Acknowledgments

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Competing interests

No competing interests to declare.

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Herpes Simplex Virus Type 1 Propagation, Titration and Single-step Growth Curves

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[Abstract] Given the endemic seroprevalence of herpes simplex viruses (HSV), its associated human diseases, and the emergence of acyclovir-resistant strains, there is a continuous need for better antiviral therapies. Towards this aim, identifying mechanistic details of how HSV-1 manipulates infected cells, how it modulates the immune responses, and how it causes diseases are essential. Measuring titers and growth kinetics of clinical isolates and viral mutants are important for a thorough characterization of viral phenotypes *in vitro* and *in vivo*. We provide protocols for the preparation as well as titration of HSV-1 stocks, and explain how to perform single-step growth curves to characterize the functions of viral proteins or host factors during infection. In particular, we describe methods to prepare and characterize high-titer HSV-1 stocks with low genome to titer ratios that are required for infection studies in cell culture and animal experiments.

Keywords: Herpes simplex virus type 1, Virus propagation, Plaque assay, Single-step growth curve

[Background] Herpes simplex virus type 1 (HSV-1) is the prototypic genus of the human pathogenic subfamily *Alphaherpesvirinae*. Worldwide, it has a seroprevalence of approximately 67% that varies from 30% to more than 90% in different human groups, depending on the age, the socio-economic status, and the geographical region (Nahmias *et al.*, 1990; reviewed in Smith and Robinson, 2002; Looker *et al.*, 2015). HSV-1 shares several features with all herpesviruses: (i) a conserved virion architecture, (ii) productive and lytic infection of cells and (iii) establishment of life-long-latent infection in the host (Whitley and Griffiths, 2002; Schleiss, 2009).

During acute infection, HSV-1 causes cutaneous and mucosal herpetic lesions, and in very young or immune-compromised individuals more severe sequelae, such as potentially blinding keratitis and life-threatening disseminated disease or encephalitis (Thompson and Whitley, 2011; Kennedy and Steiner, 2013). Despite a potent antiviral immune response inside the host, the immune system fails to clear the virus. This is achieved by HSV-1-mediated immune evasion mechanisms (Theodoridis *et al.*, 2011; Su *et al.*, 2016; Zheng, 2018; Tognarelli *et al.*, 2019), and the establishment of life-long latent infection in neurons of peripheral ganglia which are reached via retrograde axonal transport (Smith, 2012; Roizman and Whitley, 2013; Koyuncu *et al.*, 2018). During latency, viral antigen expression in the neurons is restricted, but the virus sporadically reactivates upon lowered immune surveillance and systemic stress (Padgett *et al.*, 1998; Huang *et al.*, 2011). Recurrent productive infections after reactivation comprise

anterograde axonal spread, virus release from the neurons back to mucocutaneous sites, and reinfection of epithelial cells (Enquist *et al.*, 1998; Whitley and Roizman, 2001; Diefenbach *et al.*, 2008; Smith, 2012; Koyuncu *et al.*, 2018).

Here, we describe protocols to prepare HSV-1 stocks. We generate virus stocks by harvesting post-nuclear supernatants from repeatedly freeze-thawed infected cells, or by pelleting cell-free virions released from infected cells into the conditioned medium. For cell entry and animal experiments, we recommend purifying such preparations further on sedimentation density gradients (Sathananthan *et al.*, 1997; Sodeik *et al.*, 1997; Döhner *et al.*, 2006; Dai and Zhou, 2014).

Viral stocks will maintain and acquire genomic mutations, if they provide an evolutionary advantage and are not deleterious during virus amplification. To achieve a high genomic homogeneity, a passage 1 preparation from a single viral plaque or a single clone generated by mutagenesis should be generated. Subsequent passage numbers should be kept to a minimum (Harland and Brown, 1998). We characterize virus stocks by measuring the number of plaque-forming units (pfu) by plaque-titration (see Procedure B) and the number of viral genomes by real-time detection PCR (Döhner *et al.*, 2006; Engelmann *et al.*, 2008). In addition, the particle to pfu ratio can be determined by electron microscopy (Harland and Brown, 1998; Döhner *et al.*, 2006). To this end, virus stocks are mixed with latex beads of known concentration and appropriate heavy metal salts for negative contrast, and analyzed by electron microscopy. Viral particles and latex beads are counted, and using the known concentration of the latex beads as a reference, the number of viral particles can be determined. Low ratios of genome to pfu, total protein to pfu, or particle to pfu are indicative of a high-quality virus stock.

Since HSV-1 causes strong cytopathic effects, we use plaque assays to determine the number of infectious units within a given sample. Due to the high HSV-1 prevalence, it suffices to add a pooled fraction of human IgGs to the cell culture medium to prevent virus spread via the culture medium. Alternatively, an overlay with agarose or methylcellulose could also neutralize extracellular HSV-1 virions; however, such assays require a bit advanced experimental skills. The direct cell-to-cell spread of HSV-1 leads to the formation of macroscopic plaques, which indicates the occurrence of an infectious particle present in this sample.

Titration of virus stocks is not only crucial for subsequent *in vitro* or *in vivo* infection experiments, *i.e.*, to use a defined amount of plaque forming units (pfu), but also during the analysis of viral loads in different tissues of infected animals, when comparing phenotypes of different viral mutants, or during the characterization of clinical isolates. Moreover, the impact of perturbing a host function by pharmacological inhibitors, gene silencing, genetic knock-out, or overexpression is determined by performing a time course of virus production.

The growth kinetics of viral mutants lacking essential protein functions are compared in non-complementing versus complementing cells, the latter expressing the ablated protein in *trans* (Schipke *et al.*, 2012; Sandbaumhüter *et al.*, 2013). When analyzing the impact of particular host factors on virus propagation, viral yields on deficient cells obtained by RNAi or CRISPR/Cas9 technology or from knock-out animals are compared to yields on the corresponding wildtype cells (Döhner *et al.*, 2018).

Single-step or multi-step growth curves in susceptible cell lines provide a standardized method to compare different clinical isolates or viral mutants with the respective wildtype strain, or to determine the impact of a particular host factor during infection. In single-step growth curves, a high multiplicity of infection (MOI) of 5 to 20 pfu/cell ensures complete and simultaneous infection. We recommend monitoring virus propagation up to 24 hpi during one replication cycle (Harland and Brown, 1998). In contrast, multi-step growth curves amplify the effect of more subtle phenotypes during several cycles of replication (Harland and Brown, 1998). Thus, cells are infected using a low MOI of 0.01 to 0.1 pfu/cell for 3 days. While both methods involve the same procedures, we focus here on the experimental setup for a synchronous single-step infection.

Materials and Reagents

1. Aluminum foil
2. Pipette
3. JA-17 or JA-19 centrifuge tubes (Beckman Coulter, catalog number: 357000-357007)
4. JA-17 or JA-19 rotor (Beckman Coulter, catalog numbers: 369691, 325632)
5. SW28 ultra-clear tubes (25 x 89 mm, Beckman Coulter, catalog number: 344058)
6. SW40 ultra-clear tubes (14 x 95 mm, Beckman Coulter, catalog number: 344060)
7. Cell culture flasks 175 cm², 75 cm² (from any qualified supplier)
8. 6- and 12-well cell culture plates (from any qualified supplier)
9. Cell scraper (SARSTEDT, catalog number: 83.1830)
10. Examples of permissive cells
 - a. BHK-21 cell line (baby hamster kidney, ATCC® CCL-10™)
 - b. Vero cell line (African green monkey kidney, ATCC® CCL-81™)
 - c. HeLa (human cervical cancer, ATCC® CCL-2™)

Note: Not all of these exemplary cell lines propagated further in the laboratory will turn out to be permissive for HSV-1; e.g., in our hands, certain HeLa cell lines are permissive while others are not. Thus, it is beneficial to obtain a proven permissive cell line from a laboratory working already on HSV-1, or to do some pilot experiments, before scaling up the virus preparation.

11. HSV-1(17⁺)Lox (Snijder et al., 2012) or HSV-1 strain 17⁺CMV-GFP/UL43 (BioVex; Coffin et al., 1996 and 1998)

In these reporter strains, a GFP expression cassette controlled by the CMV promoter has been inserted between the loci UL55 and UL56 or into the UL43 locus. HSV-1 belongs to biosafety class L2 for clinical isolates or S2 for genetically engineered strains, and thus any work has to be done in certified and governmentally registered L2 or S2 laboratories

12. Fetal calf serum (FCS, optimal batch for the cell line to be used for virus production; e.g., Sigma-Aldrich Chemie GmbH, catalog number: F7524)
13. Bovine serum albumin fraction V (BSA, Sigma-Aldrich Chemie GmbH, catalog number: 10735094001)

14. Bovine serum albumin, fraction V, pH 7.0 (Capricorn Scientific, catalog number: BSA-1S)
15. Pooled human immunoglobulin G (IgG, Sigma-Aldrich Chemie GmbH, catalog number: #I4506)
16. α -HSV-gD antibody (mouse monoclonal, Santa Cruz Biotechnology, catalog number: sc-21719)
17. Goat α -mouse IgG-alkaline phosphatase secondary antibody (catalog number: 115-055-166; Jackson ImmunoResearch Laboratories, Inc.)
18. DMEM cell culture medium (Lonza, catalog number: AMF4300)
19. RPMI 1640 cell culture medium (Lonza, catalog number: 12-167F)
20. PBS (Lonza, catalog number: 17-512F)
21. CO₂-independent medium (Thermo Fisher Scientific, catalog number: 18045-054)
22. HEPES (from any qualified supplier)
23. Penicillin-Streptomycin mixture (from any qualified supplier)
24. L-Glutamine (from any qualified supplier)
25. Trypsin-EDTA (from any qualified supplier)
26. MEM non-essential amino acids (from any qualified supplier)
27. Nycodenz (Axis-Shield PoC, catalog number: 1002424)
28. Methanol (from any qualified supplier)
29. Ethanol (from any qualified supplier)
30. CaCl₂ (from any qualified supplier)
31. NaOH (from any qualified supplier)
32. Tris (from any qualified supplier)
33. Tris-HCl (pH 9.5)
34. NaCl (from any qualified supplier)
35. 2-(N-morpholino) ethane sulfonic acid (MES, Sigma-Aldrich Chemie GmbH, catalog number: M5287)
36. Trypan Blue solution 0.4% (from any qualified supplier)
37. Liquid N₂
38. Aqua bidest
39. DAPI (4',6-diamidino-2-phenylindole, from any qualified supplier)
40. NP-40 (from any qualified supplier)
41. DMSO (dimethyl sulfoxide, from any qualified supplier)
42. Nitroblue tetrazolium chloride (from any qualified supplier)
43. 5-bromo-4-chloro-3-indolyl phosphate (from any qualified supplier)
44. MgCl₂ (from any qualified supplier)
45. Cell culture medium (see Recipes)
46. Formalin (see Recipes)
47. TSM (see Recipes)
48. Inoculation medium (1) (see Recipes)
49. Inoculation medium (2) (see Recipes)
50. MNT buffer (see Recipes)

51. Crystal violet (from any qualified supplier, see Recipes)
52. Paraformaldehyde (PFA; from any qualified supplier, see Recipes)
53. Nuclei stain stock solution (see Recipes)

Equipment

1. Beckman Optima L-90K ultracentrifuge (Beckman Coulter, catalog number: COL11F05)
2. Megafuge 2.0 RS (Heraeus, catalog number: 75015505)
3. Rocking Platform wt 15 (Biometra, catalog number: 042-590)
4. Cell Imaging System (e.g., EVOS FL AMG/Life Technologies, catalog number: AMF4300)
5. Perfect Spin 24R refrigerated microcentrifuge (Peqlab, catalog number: C2500R-PL-230)
6. HERAcell 150i CO₂ Incubator (Thermo Fisher Scientific, catalog number: 51026282)
7. Neubauer chamber (from any qualified supplier)
8. Ultrasound bath Sonorex RK 100 (Bandelin Elektronik GmbH & Co., catalog number: 66253)
9. Cup sonifier Sonopuls UW 3200 (Bandelin Elektronik GmbH & Co., catalog number: 364000064495003)
10. Biocomp gradient master Model 106 (BioComp Instruments, catalog number: 613; <http://biocompinstruments.com/our-approach/gradient-forming>)
11. Fume hood

Procedure

A. Virus propagation

Virus propagation can be scaled up to 40 T₁₇₅ cell culture flasks. For virus propagation, we recommend using BHK-21 cells, although several other permissive cell lines such as Vero or HeLa cells can be used as well. HSV-1 mutants lacking the expression of essential proteins are propagated on appropriate complementing cell lines.

Use a passage 1 virus isolate, clone or mutant to generate a medium-scale passage 2 virus stock that provides the inoculum for a large-scale preparation of passage 3 to 4 for infection experiments. To produce virus stocks, cells should be infected at a low multiplicity of infection (MOI). This fosters amplification and packaging of complete viral genomes, and prevents an enrichment of viral particles harboring defective genomes (Harland and Brown, 1998).

1. Three days prior to inoculation, seed BHK-21 cells into five cell culture flasks (T₁₇₅) in 30 ml cell culture medium (10% FCS; Recipe 1) for confluence two days after (= one day prior to infection).
2. One day prior to inoculation, split BHK-21 cells approximately 1:3 into 16 cell culture flasks (T₁₇₅) in 30 ml of fresh cell culture medium (10% FCS) to achieve approximately 90 to 95% confluence on the next day. Alternatively, seed cells at lower cell density (1:10 to 1:20) three days prior to inoculation to achieve approximately 90 to 95% confluence on the day of inoculation. However,

to facilitate achieving an appropriate cell density, we recommend seeding cells at higher density one day prior to inoculation.

Note: Use 15 cell culture flasks for virus inoculation and one cell culture flask for determining the cell density to calculate the amount of the HSV-1 inoculum. The latter flask can be used for further cell culture.

- a. One day prior to inoculation, aspirate the cell culture medium and wash cells with 10 ml PBS/cell culture flask.
- b. Aspirate PBS, immediately add 3 ml of trypsin/EDTA, ensure complete distribution on the cell layer and incubate at 37 °C for 3-4 min.
- c. Meanwhile, prepare 16 T₁₇₅ cell culture flasks each containing 27 ml pre-warmed cell culture medium.
- d. Microscopically verify trypsinization of the cells. When cells are detached from the bottom of the cell culture flask, add 7 ml cell culture medium/flask and resuspend cells by pipetting up and down.
- e. Pool 10 ml cell suspensions from each of the five cell culture flasks into one 50 ml tube.
- f. Gently add 3 ml cell suspension to the 16 cell culture flasks to reach a final volume of 30 ml/flask.
- g. Mix cell suspension by gently moving the cell culture flasks vertically, horizontally and diagonally.
- h. Place cell culture flasks into a humidified incubator at 37 °C and 5% CO₂.

Note: If your incubator has uneven shelves, place cell culture flasks for 10 min on a planar bench to achieve a more uniform cell seeding. Do not leave cells too long outside the incubator, since the pH of the DMEM medium will drop in the absence of an additional buffer substance or CO₂.

- i. Grow cells for one day until they reach approximately 90 to 95% confluence (Figure 1).
Note: The density of optimally cultured cells influences virus yields, as subconfluent cells are more susceptible to HSV-1 than confluent ones. If seeded at low density, some cell lines tend to grow to small confluent cell islets. Cells in the periphery of such islets are more susceptible to HSV-1 infection than confluent cells in a monolayer, since the major HSV-1 receptor nectin-1 is sequestered into cell-cell adhesion contacts and not accessible for virus binding (Schelhaas et al., 2003; Marozin et al., 2004; Snijder et al., 2012).

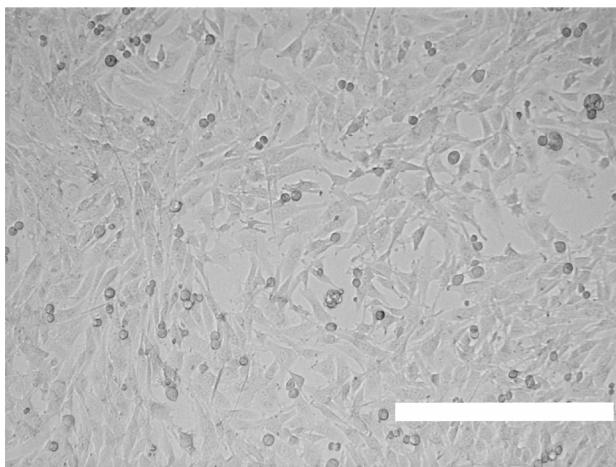


Figure1. Subconfluent BHK-21 cells for HSV-1 inoculation. Scale bar = 0.4 mm.

3. At the day of virus propagation, inoculate BHK-21 cells with the respective HSV-1 strain or mutant.
 - a. Wash one T₁₇₅ cell culture flask with 10 ml PBS, aspirate, trypsinize with 3 ml trypsin/EDTA as described in Step A2b.
 - b. Resuspend cells in 25 ml of medium and count the number of cells using a Neubauer chamber or an alternative method.

Note: Typically, the cell amount per T₁₇₅ cell culture flask is approximately 1.5 x 10⁷ to 3 x 10⁷ cells. Use these cells for further cell culture.

- c. Calculate the volume of a passage 2 virus stock required to infect 15 T₁₇₅ cell culture flasks with a multiplicity of infection (MOI) of 0.01 pfu/cell using the formula below:

Formula:

$$\text{volume of HSV p2 } [\mu\text{l}] = \frac{\left(\frac{\text{cells}}{\text{flask}}\right) * X \text{ flasks} * \text{MOI } [\frac{\text{pfu}}{\text{cell}}]}{\text{titer of HSV p2 } [\frac{\text{pfu}}{\mu\text{l}}]}$$

Example calculation: Titer of stock preparation HSV-1 p2: 6 x 10⁸ pfu/ml = 6 x 10⁵ pfu/μl
MOI of 0.01 pfu/cell; 40 flasks with 2 x 10⁷ cells/flask

$$\text{volume of HSV p2 } [\mu\text{l}] = \frac{\frac{2 \times 10^7 \text{ cells}}{\text{flask}} * 40 \text{ flasks} * 0.01 \frac{\text{pfu}}{\text{cell}}}{6 \times 10^5 \frac{\text{pfu}}{\mu\text{l}}} \\ = 13.33 \mu\text{l}$$

- d. Prepare one inoculum for all flasks by diluting the calculated virus volume into RPMI 1640 supplemented with 20 mM HEPES (use 5 ml/flask). Alternatively, dilute calculated virus in CO₂-independent medium supplemented with 0.1% (w/v) cell culture grade BSA (use 5 ml/flask). Both media warrant a stable pH during subsequent inoculation of the cells outside a CO₂ incubator.

Note: As CO₂ is a gas, it evaporates quickly from the medium outside a CO₂ incubator, which will therefore turn more basic. While cells recover quickly from a short-term incubation in a more alkaline medium, HSV-1 is very sensitive to higher pH. Already at pH 7.6, the titer of a given virus suspension drops significantly.

- e. Harvest cell culture medium from each cell culture flask.

Note: To reduce the costs for virus preparation, the cell culture medium removed from the flasks can be pooled in a sterile flask, placed in a 37 °C incubator and added to the BHK-21 cells after inoculation.

- f. Prior to infection, wash cells once with 10 ml/cell culture flask PBS, aspirate, and immediately add 5 ml inoculum.

Note: Wash and infect up to five cell culture flasks simultaneously.

- g. Inoculate cells on a gently moving rocking platform at room temperature (RT) for 1 h. Ensure that the cells are completely covered by the inoculum.

- h. After 1 h, add 25 ml/flask (preserved) cell culture medium.

- i. Incubate HSV-1-infected BHK-21 cells at 37 °C and 5% CO₂ for three to four days.

4. Harvest extracellular HSV-1 virions.

Note: Microscopically monitor the infected cells daily (Figure 2). In our hands, the time point of harvesting is crucial and can vary from three to four dpi. For good yields of high-quality HSV-1 extracellular virions, harvest the cell culture supernatants when 80 to 90% of the cells display cytopathic effects, i.e., cells are rounded up and detach from the bottom of the flask. When harvesting HSV-1 too early, the yields for extracellular virus will be lower. When harvesting HSV-1 too late, the virus stocks will be of lower quality due to cell debris. In particular, released DNA/RNA will crosslink many viral particles and thus hampers sufficient resuspension of aggregated virions (see Step A4d).

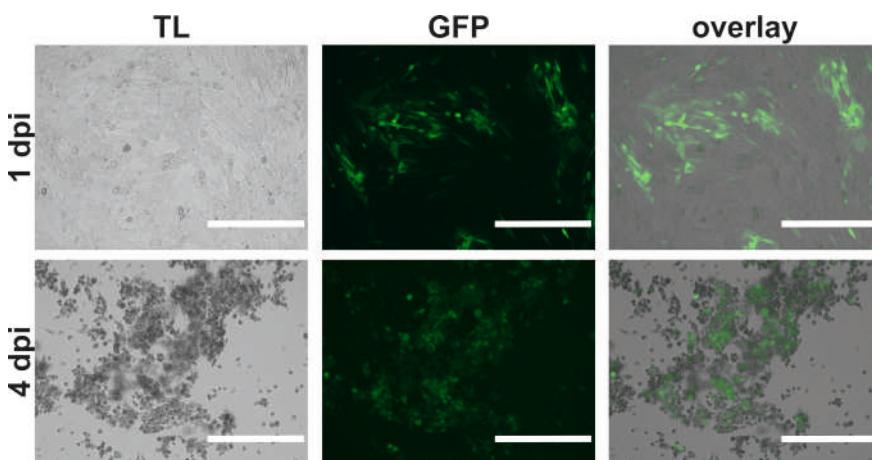


Figure 2. BHK-21 cells infected with HSV-1 strain 17+CMV-GFP/UL43 at 1 dpi (upper panel) and 4 dpi (lower panel). Scale bar= 0.4 mm.

- a. At three or four dpi, detach cells by tapping the cell culture flask, rinse remaining cells from the bottom and collect the cell-virus suspension in 50 ml tubes.
- b. To pellet the cells centrifuge at 1,900-2,850 $\times g$ and 4 °C for 10 min.
- c. The sedimented infected cells can be subsequently used to isolate nuclear capsids (Wolfstein et al., 2006; Bucks et al., 2007; Radtke et al., 2010), or infectious virions from the cytoplasm by freeze-thaw cycles. For this, resuspend the cell pellets in an appropriate small volume of MNT buffer (Recipe 8), freeze in liquid N₂, and store at -80 °C.
- d. Transfer the supernatant containing HSV-1 virions to precooled centrifuge tubes. Centrifuge in a JA-17 rotor at 4 °C and 17,000 rpm (maximal 39,813 $\times g$) for 2 h, or in a type 19 rotor at 4 °C and 12,000 rpm (maximal 21,500 $\times g$) for 90 min. While the first centrifugation setting warrants a higher yield of pelleted virions, the latter one warrants easier and more homogenous resuspension of the virus pellet to single virions.

Note: After centrifugation, label the positions of the pellets on the outside of the tubes.

- e. Place centrifuge tubes on ice, aspirate supernatants completely, but carefully, and add 200 to 500 μl /tube MNT buffer to resuspend the pellets.

Note: We recommend using MNT buffer instead of PBS for storage of HSV-1 stocks since sodium phosphate salts precipitate upon cooling causing dramatic pH changes. MES and Tris-HCl maintain a more stable pH during the freezing procedure (Gómez et al., 2001).

- f. Tilt tubes for complete coverage of the virus pellet with MNT buffer and allow virus pellets to swell at 4 °C over-night. Alternatively, resuspend the pellets in MNT buffer by pipetting, but avoid the formation of foam or bubbles. Transfer the resuspended pellets, for sonication on the next day, to a fresh glass vial coated with BSA (fraction V or cell culture grade) to prevent particle loss, due to unspecific binding to the glass surface. Close the vial tightly with parafilm and allow viral particles to swell at 4 °C for 24 to 36 h. This glass vial will be used for sonication on the next day (see Step A5b).

5. Resuspend virus suspension.

- a. On the next day, pool and/or further resuspend the medium pellet. Work on ice and avoid generating foam and bubbles.
- b. We recommend further homogenizing the medium pellet in an ultrasonic bath filled with ice water (pulse three times for 30 s with 30 s pauses in between). If necessary, repeat Step A5b two to three times until the virus suspension appears homogenous.
- c. Aliquot the resuspended medium pellet virus for storage into small volumes (25-100 μl , depending on the experiments planned) into sterile screw cap vials, snap-freeze in liquid N₂, and store at -80 °C. To prepare gradient-purified virus stocks, skip this step and directly proceed with the purification protocol (see Step A6).

Note: Avoid repeated freeze-thaw cycles of virus stocks. Always store stocks at -80 °C, since they continuously lose infectivity at -20 °C. Aliquoted vials should be snap-frozen rapidly in liquid N₂, thawed quickly and kept at 0 to 4 °C until use.

6. Gradient purify HSV-1 stocks.

Virus stocks for virus entry or animal experiments should be gradient-purified to remove residual cell debris and cytokines that might have been synthesized during infection. HSV-1 virions can be purified using sucrose, Ficoll, nycodenz or iodixanol (Opti-prep) gradients (Sathananthan *et al.*, 1997; Sodeik *et al.*, 1997; Döhner *et al.*, 2006; Marconi and Manservigi, 2014). At the same density, Ficoll, nycodenz and iodixanol solutions have a lower osmotic pressure than sucrose solutions. When HSV-1 virions enter a sucrose sedimentation gradient, they lose water due to an increasing osmotic pressure at higher sucrose concentration, and more importantly swell abruptly upon dilution from their sucrose fraction into MNT or PBS buffers. Such changes in osmotic pressure impair the physical integrity of enveloped virions. Therefore, we developed protocols using nycodenz for gradient purification of HSV-1 stocks (Döhner *et al.*, 2006).

- a. Prepare a linear nycodenz gradient. Pipette MNT buffer supplemented with 10% nycodenz into ultraclear tubes up to half height and label fill level. Carefully underlay with a 40% nycodenz in MNT using a syringe attached to a long needle until the interphase is at the half-full line (Video 1).



Video 1. Visualization of the interphase formation during preparation of a step gradient.

The tube contains 10% sucrose (transparent). We underlay with the 40% sucrose (blue) using a syringe. To better visualize the interphase formation, we stained the 40% sucrose solution here with a blue dye. If preparing gradients for virus preparations we do not add any dye. It is crucial to add the heavy solution smoothly without pressing the needle to the bottom of the tube and without pushing the plunger too abruptly. An interphase between the 10% and 40% solution forms and migrates up.

- b. If using a Biocomp gradient master: Close tubes with corresponding caps, level the gradient master (see manual) and prepare a linear gradient using the parameters provided by the vendor. Place the tubes with the step gradient into the tube holder, place the tube holder onto the magnetic platform of the gradient master and start the program. The platform will tilt and then rotate. The tilting angle (°), the speed (rpm), and duration of rotation (min:s) are

specified by the user as indicated in the table below (Table 1). For better visualization, we refer to <https://www.youtube.com/watch?v=wizxYB5brz8>.

Table 1. Centrifugation settings for the Gradient master

Rotor*	Step	Time [min:s]	Angle	Speed	Temperature
SW28	1	7:00	50°	25 rpm	RT
	2	0:15	80°	15 rpm	RT
SW40	1	1:11	80°	25 rpm	RT

*SW28 for larger or SW40 for medium volumes.

c. **Alternative procedure:** Prepare a two-step gradient as described in Step A6a and seal tightly. To receive a linear gradient via diffusion, gently place the tube horizontally, stabilize and keep at RT for 90 min.

d. If necessary, discard the respective sample volume from the top of the gradient. Subsequently, overlay the gradient with 1 ml and 2-3 ml of resuspended medium pellet for SW40 and SW28 gradient, respectively.

Note: Avoid overloading of the gradients.

e. Centrifuge in a pre-cooled ultracentrifuge (e.g., Beckman Optima L-90K) at 4 °C. When using the SW28 rotor, centrifuge at 20,000 rpm (maximal 72,128 x g) for 105 min, when using the SW40 rotor, centrifuge at 20,000 rpm (maximal 71,142 x g) for 120 min.

Note: If using other settings or rotors, recalculate speed and time. Choose the appropriate rotor depending on the volume of virus suspension to be gradient-purified.

f. Label the light-scattering virus band slightly beneath the middle of the gradient (Figure 3). Remove the upper gradient solution and harvest the light-scattering band with a yellow tip pipette without disturbing the gradient. We harvest 1 to 4 ml gradient-purified virus from one tube of an SW28 bucket. Transfer the harvested band into a vial, mix gently by pipetting, prepare single-use aliquots (10 to 100 µl, depending on purpose) in autoclaved 0.5 ml screw caps with O-rings, snap-freeze in liquid nitrogen, and store at -80 °C. The given volume depends on the volume that had been layered onto the gradient, and whether one aims for a highly concentrated stock for cell entry experiments, or complete yield for experiments performed at a lower MOI.



Figure 3. Gradient purification of HSV-1 virions. Exemplary result of a linear nycodenz sedimentation gradient with a light-scattering virus band (arrow) slightly beneath the middle of the tube (red line).

B. Plaque Assay

Note: We recommend technical duplicates for each virus stock. When comparing different viruses in single-step growth kinetics (e.g., parental vs. mutant or clinical isolates), perform plaque assays in parallel including three biological and two technical replicates for each sample.

1. Prepare one T₇₅ cell culture flask of confluent Vero cells for 6-well or 12-well plate plaque assay.
Note: One T₇₅ cell culture flask with confluent Vero cells is sufficient for seeding two 6-well or three 12-well plates.
2. One day prior to inoculation, seed Vero cells from one confluent T₇₅ cell culture flask into one 6-well plate at 3×10^5 to 5×10^5 cells/well or 12-well plate at 1.5×10^5 to 2.5×10^5 cells/well to achieve just confluence on the following day.
 - a. Add 5 ml PBS, gently wash cell layer and aspirate PBS.
 - b. Immediately add 1.5 ml of trypsin/EDTA, ensure even distribution on the entire cell layer and incubate at 37 °C for 5 min.
 - c. Microscopically monitor cell rounding. When cells are completely detached, add 8.5 ml of prewarmed medium and resuspend cells by pipetting up and down.
 - d. Count the cells using a Neubauer chamber or an alternative method.
 - e. Prepare an appropriate volume of medium to achieve a final concentration of 0.5×10^6 cells/ml, and transfer 2 ml/6-well or 1 ml/12-well into each well.
 - f. Incubate Vero cells at 37 °C and 5% CO₂ for 16 to 20 h to reach just-confluence.
3. At the day of inoculation, prepare serial 10-fold dilutions of the virus stocks to be titrated.

- a. Prepare 30 ml of inoculation medium (Recipes 6 and 7): RPMI 1640 supplemented with 20 mM HEPES and 0.1% (w/v) BSA or CO₂-independent medium supplemented with 0.1% (w/v) cell culture grade BSA. Both media maintain a stable pH while inoculating the cells outside a CO₂ incubator on a rocking platform.
Note: For an uncharacterized virus stock, use dilutions ranging from 10⁻² to 10⁻⁹ (see Figure 4). For an uncharacterized sample of a growth curve, use dilutions ranging from 10⁻² to 10⁻⁶. If prior experiments permit an estimation of the titer, it suffices to plate fewer dilutions. Do not forget an uninfected mock control.
- b. Thaw a virus stock and prepare a 10⁻² dilution by adding 10 µL of the stock to 990 µL of inoculation medium and vortex thoroughly.
- c. Prepare the 10⁻³ dilution by transferring 130 µL of the 10⁻² dilution with a fresh pipette tip to 1,170 µL inoculation medium. Vortex.
- d. Complete this serial dilution until you reach the desired final dilution (see Figure 4).

	10 µL	130 µL	130 µL	130 µL	130 µL	130 µL	130 µL	130 µL
dilution	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹
infection medium	990 µL	1170 µL	1170 µL	1170 µL	1170 µL	1170 µL	1170 µL	1170 µL

Figure 4. Pipetting scheme of a serial dilution for plaque assays

- e. Gently wash Vero cells once with 1.5 ml/well inoculation medium.
 - f. Add 500 µL/6-well or 400 µL/12-well of the 10⁻⁵ to 10⁻⁹ dilutions to titer a virus stock, or the 10⁻² to 10⁻⁶ dilutions for samples of a growth curve to the designated well. Perform in single-sample or in duplicates and incubate cells on a gently rocking platform at RT for 1 h.
 - g. At 1 hpi, aspirate the inoculum and add 2 ml/6-well or 800 µL/12-well of medium freshly supplemented with pooled human IgG.
Caution: As the titers of neutralizing antibodies vary among the human population, we titrate every batch by pretreating a standard HSV-1 stock with 2-fold serial dilutions of a pooled human IgG stock solution. An IgG concentration of about 10 to 20 µg/ml usually suffices to neutralize a virus stock diluted for plaque assays. It may require a higher concentration of human IgGs to neutralize a synchronous infection at a higher MOI.
 - h. Incubate cells at 37 °C and 5% CO₂ for three days.
4. At 2 dpi, microscopically analyze plaque formation (Figure 5). A fluorescent microscope could be used to monitor cells infected with a strain expressing a fluorescent protein.

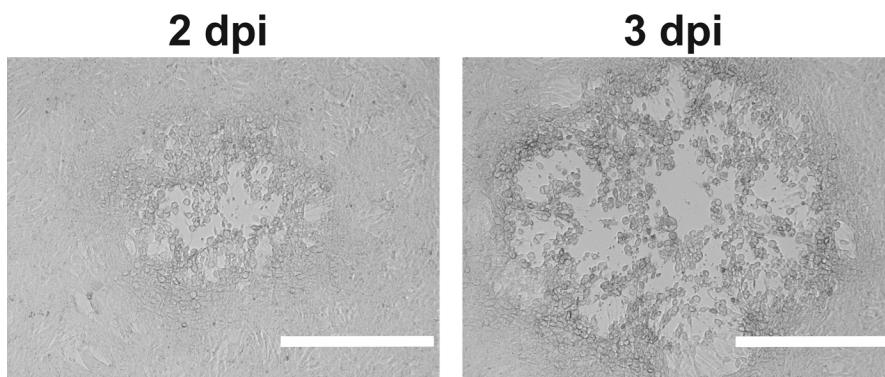


Figure 5. HSV-1 plaques in a Vero cell monolayer at 2 dpi (left) or 3 dpi (right). Scale bar = 0.4 mm.

5. Variant 1: At 3 dpi, stain the cells with crystal violet.
 - a. Aspirate the medium from each well, and fix the cells using 1 ml/6-well or 400 μ l/12-well of 9% formalin (Recipe 3) at RT for 10 min. Alternatively, fix cells using pre-cooled (-20 °C) 100% methanol for 3 min.

Note: Carefully add the fixative to the wall of the wells to avoid cell detachment.
 - b. Aspirate the fixative, and, in the case of methanol fixation, air-dry the wells for at least 5-10 min. Add 1 ml/6-well or 400 μ l/12-well of 0.1% (w/v) crystal violet (Recipe 4) staining solution, and incubate at RT for 10 min.
 - c. Aspirate staining solution, wash twice with 1 ml/well Aqua bidest and let dry (Figure 6).

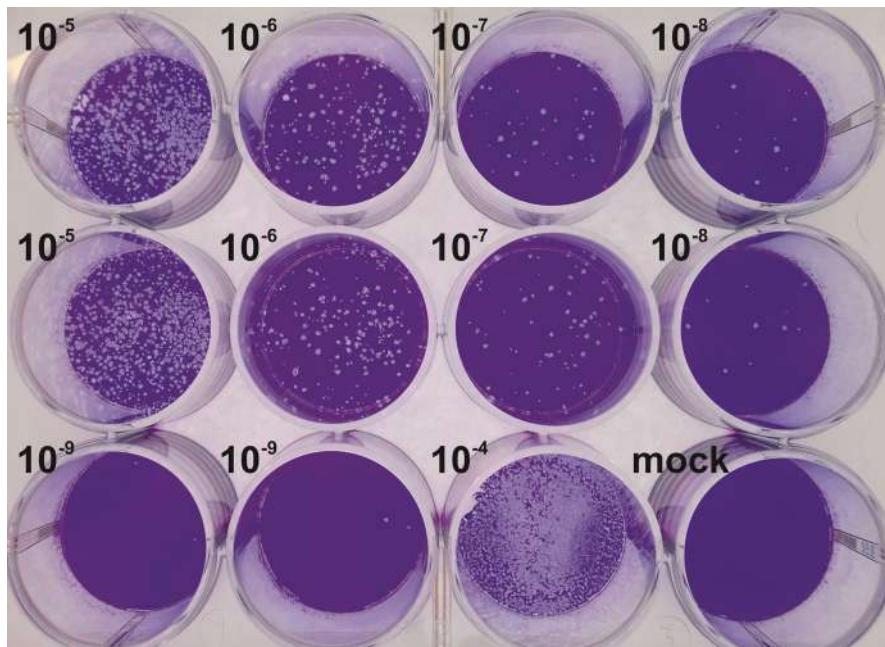


Figure 6. Crystal violet stained HSV-1 plaque assay. Vero cells cultured in a 12-well plate were inoculated with 10-fold serial dilutions of a virus suspension, fixed at 3 dpi and stained with crystal violet.

6. Variant 2, for small plaques in a 6-well plate: At 2 dpi, label infected cells with an antibody directed against the HSV-1 envelope glycoprotein D (gD; Döhner et al., 2002).
 - a. Aspirate the medium from each well and fix cells with -20 °C pre-cooled methanol at RT for 3 min.
Note: Carefully add the fixative to the wall of the wells to avoid cell detachment.
 - b. Aspirate the fixative, air-dry the cells and incubate on a gently rocking platform at RT for 30 min with 0.5 ml/well PBS supplemented with 10% FCS (PBS/FCS) to block unspecific protein binding.
 - c. Replace PBS/FCS with 0.5 ml/well primary α -gD antibody diluted in PBS/FCS and incubate on a rocking platform at RT for 30 min. We use our present batch of the anti-gD antibody (mAb DL6) at a dilution of 1:2,000. Since antibody concentrations vary among suppliers and batches, we recommend titrating each batch using dilutions ranging from 1:50 to 1:5,000.
 - d. Remove the primary antibody and wash cells three times with 2 ml/well PBS for 10 min.
 - e. Replace PBS with 0.5 ml/well of secondary goat α -mouse IgG coupled to alkaline phosphatase in PBS/FCS and incubate on a rocking platform at RT for 30 min. We use our present batch of the alkaline phosphatase-coupled anti-mouse IgG antibody at a dilution of 1:2,000. Since antibody concentrations vary among suppliers and batches, we recommend titrating each batch using dilutions ranging from 1:500 to 1:10,000.
 - f. Remove the secondary antibody and wash three times with 2 ml/well PBS for 10 min, and two times with 2 ml/well TSM (Recipe 5) for 5 min each.
 - g. Replace with 0.5 ml/well of 0.2 mM nitroblue tetrazolium chloride and 0.8 mM 5-bromo-4-chloro-3-indolyl phosphate in TSM buffer. Protect from light with aluminum foil, and incubate on a rocking platform at RT until dark plaques appear.
 - h. Replace staining solution, and rinse the wells twice with Aqua bidest to stop alkaline phosphatase reaction by lowering the pH and removing the substrate.
 - i. Aspirate water and air-dry the plates (Figure 7).

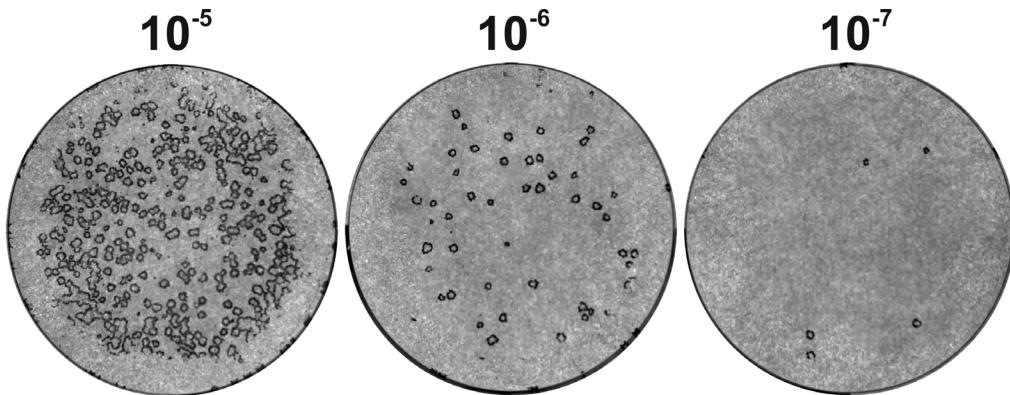


Figure 7. α-gD stained HSV-1 plaque assay. Vero cells were inoculated with serial dilutions of HSV-1 suspensions and infected for 3 days. Cells were fixed and subsequently labeled with an α-gD antibody followed by labeling with a secondary alkaline phosphatase antibody.

7. Count the plaques in wells containing approximately 20 to 50 plaques in a 6-well or 10 to 40 in a 12-well.

Note: In wells with too many plaques the titer is underestimated as fused plaques will be recorded as only one plaque. In contrast, in wells with few plaques the titer is overestimated as they may contain irregularities in the cell monolayer that may be interpreted falsely as plaques.

8. Calculate the titer in pfu/ml using these formulas:

6-well plate:

$$\text{pfu / ml} = \frac{\text{number of plaques per well}}{\text{dilution * infection volume (0.5 ml)}}$$

12-well plate:

$$\text{pfu / ml} = \frac{\text{number of plaques per well}}{\text{dilution * infection volume (0.4 ml)}}$$

Example calculation: Titration in 6-well plates in duplicates, using 0.5 ml of the inoculum.

Assumption: Wells treated with the 10^{-8} dilution of the inoculum show plaques in the range of 20 to 50, namely 28 and 32.

$$\frac{\text{pfu}}{\text{ml}} = \frac{\frac{28+32}{2} \text{ pfu}}{10^{-8} * 0.5 \text{ ml}} = 6 * 10^9 \frac{\text{pfu}}{\text{ml}}$$

C. Single-step growth kinetics

Note: Measuring samples from at least three biological independent experiments once is better than measuring samples from a single experiment in triplicates. We usually measure samples from at least three independent experiments in duplicates.

1. One day prior to infection, seed susceptible cells, e.g., Vero, into 6-well plates at 0.25×10^6 cells/well in 2 ml medium, and incubate at 37°C and 5% CO_2 for 16 to 20 h.

Note: The cells should be 90 to 95% confluent at the time of inoculation for single-step growth kinetics. For each virus isolate, virus mutant, cell line, and time point, one well is required to monitor virus propagation, and another one to estimate the cell density.

2. On the next day, remove the medium and wash the cells three times with 1 ml/well PBS.
3. Assess the cell density in one well of 6-well plate by performing DAPI staining.
 - a. Fix cells with 1 ml/well 3% paraformaldehyde (PFA) in PBS at RT for 20 min and wash three times with 1 ml/well PBS.
 - b. Prepare a 1:200 dilution of nuclei stain stock solution in PBS supplemented with 0.1% (v/v) Triton X-100, and stain the cells with 1 ml/well at RT for 10 min.
 - c. Remove staining solution and carefully wash the cells three times with 1 ml/well PBS. Measure the cell density by automated fluorescence microscopy. There are several

automated fluorescence microscopes available with proprietary software to obtain cell number per area. At present, we use a Cytation 3 imaging reader (BioTek, Agilent, Winnoski, VT, US).

4. Prepare virus inoculum.
 - a. Calculate the volume of the inoculum for all wells, and dilute the virus stock to yield approximately 5 to 10 pfu/cell with CO₂-independent medium supplemented with 0.1% (w/v) cell culture grade BSA. Prepare one sample CO₂-independent medium lacking virus as mock-treated control.
 - b. Inoculate or mock-treat cells with 1 ml/well, and incubate cells on a rocking platform at RT for 1 h.
 - c. At 1 hpi, wash cells three times with 1 ml/well PBS, and incubate with 2 ml/well medium supplemented with 1% FCS at 37 °C and 5% CO₂. This is 0 h of the time scale.
5. Determine the amount of extracellular and cell-associated intracellular virus at various time points, e.g., in 3 h intervals from 3 to 24 hpi.
 - a. To determine extracellular viral yields, transfer supernatants from a 6-well plate into 2 ml tubes kept on ice. Centrifuge at 4 °C and 150 x g for 5 min to pellet cells.
 - b. Meanwhile, add 600 µl/well MNT buffer to prevent cells from drying out.
 - c. After centrifugation, harvest supernatants, aliquot for plaque assay (6 x 300 µl), snap-freeze in liquid N₂, and store at -80 °C. Keep the resulting cell pellets on ice.
 - d. To determine intracellular viral yields, harvest residual Vero cells of each well by scraping, and combine with the cells harvested from the medium (Step C5c).
 - e. Transfer 600 µl of this cell suspension to fresh 2 ml tubes.
 - f. Add 400 µl MNT buffer to each well, to wash the well and scraper, and transfer this also to the respective 2 ml tube (Step C5e) reaching a final volume of 1 ml.
 - g. Vortex cell suspensions, snap-freeze in liquid N₂ and store at -80 °C.
 - h. To release the cell-associated virus, the cells are subjected to three cycles of freeze-thaw, or sonicated in an ultrasound bath until the solution appears clear.
 - i. Centrifuge these suspensions at 4 °C and 150 x g for 3 min, aliquot the supernatants in 4 x 200 µl, snap-freeze in liquid N₂, and store at -80 °C. Furthermore, store the 2 ml vials with residual 200 µl containing any potential pellets.
6. Determine the intra- and extracellular viral yields by plaque assay (see Procedure B).

Note: In addition, the number of intra- and extracellular viral genomes can be determined by real-time detection PCR using a standard diagnostic probe (e.g., Engelmann et al., 2008).

Data analysis

When comparing different viruses in single-step growth kinetics (e.g., parental vs. mutant or clinical isolates), perform plaque assays in parallel including three biological and two technical replicates

for each sample. For some statistical considerations on virological experiments, please refer to Richardson and Overbaugh (2005) and Wang and Bushman (2006).

Notes

Primary risks for people working with HSV are due to droplet exposure of mucous membranes of the eyes, nose or mouth, inhalation of concentrated aerosols, or accidental injection. Lab workers thus require a health check, and regular intensive training in biosafety L2/S2 work. Working with HSV presupposes an approved biosafety level S2 laboratory. Handling of virus preparations needs to be performed in a biosafety L2/S2 cabinet, while wearing gloves and goggles. We avoid the use of needles, syringes, or any other sharp devices to prepare high-titer virus stock solutions. All waste that contains or has encountered infectious virus needs to be inactivated either chemically (bleach or aldehyde-containing disinfections), or by autoclaving for at least 20 min at 121°C, according to the biosafety L2/S2 regulation in the respective country and the L2/S2 guidelines of the respective institution. We also recommend to UV-irradiate all waste as well as the biosafety L2/S2 cabinet after working with virus-containing samples.

Recipes

1. Cell culture medium (store at 4 °C, use within 3 months after opening and before expiry date)
DMEM supplemented with:
100 mg/ml Streptomycin (optional)
100 U/ml Penicillin (optional)
2 mM L-glutamine
1x of MEM nonessential amino acids solution (100x)
1% or 7.5-10% FCS (optimized for the cell line to be used for propagation)
2. 3% paraformaldehyde in PBS, pH 7.4 (store at -20 °C, stable for at least 6 months)
 - a. Mix 12 g paraformaldehyde with 40 ml 10x PBS, fill up to 300 ml with Aqua bidest
 - b. To dissolve the PFA, heat up to maximal 60 °C (fume hood) while stirring continuously
 - c. Add 40 µl of 1 M CaCl₂ and 40 µl of 1 M MgCl₂
 - d. After the PFA is dissolved, cool the solution down to RT, adjust the pH to 7.4 using 1 M NaOH and fill up to 400 ml with Aqua bidest
3. Formalin (store at RT, stable for at least 1 month)
Dilute a 36.5% stock solution 1:4 in PBS to reach a final concentration of 9% formaldehyde
4. Crystal violet (store at RT, stable for at least 1 month)
 - a. Prepare a 5% stock solution by dissolving 0.25 g crystal violet in 5 ml 100% ethanol (store at RT, stable for at least 6 months)
 - b. Prepare a working solution by 1:50 dilution in Aqua bidest to reach a final concentration of 0.1% crystal violet in 2% ethanol
5. TSM buffer (store at RT, stable for at least 1 month)

- 100 mM Tris-HCl, pH 9.5
100 mM NaCl
5 mM MgCl₂
6. Inoculation medium (1) (store at 4 °C, use within 3 months after opening and before expiry date)
RPMI 1640
20 mM HEPES
0.1% (w/v) cell-culture grade BSA
7. Inoculation medium (2) (store at 4 °C, use within 3 months after opening and before expiry date)
CO₂-independent medium
20 mM HEPES
0.1% (w/v) cell-culture grade BSA
8. MNT buffer (filter sterilize and store at RT, stable for at least 12 months)
30 mM MES
100 mM NaCl
20 mM Tris
9. Nuclei stain stock solution (store at 4°C, protect from light, stable for at least 12 months)
10 mg/ml DAPI
10% (v/v) DMSO
0.1% (v/v) NP-40
5% (w/v) BSA fraction V
10 mM Tris-HCl, pH 7.4
146 mM NaCl
22 mM CaCl₂
22 mM MgCl₂
Dilute 1:200 for a working solution

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Competing interests

The authors declare no competing interests.

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Protocol for Ribosome Profiling in Bacteria

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[Abstract] Ribosome profiling provides information on the position of ribosomes on mRNA on a genomic scale. Although this information is often used to detect changes in gene expression under different conditions, it also has great potential for yielding insight into the mechanism and regulation of protein synthesis itself. First developed in yeast, ribosome profiling involves the isolation and sequencing of ribosome-protected mRNA fragments generated by nuclease treatment. Since the application of ribosome profiling in bacteria has been problematic, we report here a systematically optimized protocol for *E. coli* that we have used with success for other bacteria as well. Cells are harvested by flash-freezing cultures directly in liquid nitrogen. After lysis, translation is arrested by high magnesium concentration without the use of antibiotics. These improvements eliminate artifacts induced by harvesting cells by centrifugation or filtration and by use of chloramphenicol to arrest translation. These improvements are especially appropriate for studies where the exact position of the ribosome is critical, and not merely the number of ribosomes per message, such as studies aimed at monitoring differences in local elongation rates.

Keywords: Ribosome profiling, Ribosome pausing, Translational efficiency, Gene expression, Bacteria

[Background] mRNAs are translated at varying efficiencies depending on properties inherent to the mRNA such as initiation rates, mRNA structure, and codon usage. In addition, physiological changes in the cell can alter how efficiently mRNAs are translated. Ribosome profiling was developed to monitor gene expression at the level of protein synthesis, making it possible to ask global questions about which regions of the genome are translated, when they are translated, and how translation is regulated. First developed by Ingolia and Weissman in yeast (Ingolia *et al.*, 2009), ribosome profiling utilizes nuclease digestion to degrade regions of mRNA that are not occupied by ribosomes, generating ribosome-protected mRNA fragments that are isolated, cloned, and sequenced. When the reads are mapped back to the genome, they reveal the position of ribosomes throughout the transcriptome, providing a powerful tool to monitor translation and its regulation *in vivo*. Our focus here is the application of ribosome profiling to bacteria, first reported by Weissman and Bukau (Oh *et al.*, 2011; Li *et al.*, 2012; Becker *et al.*, 2013). Our protocol is derived from these pioneering studies and incorporates recent improvements described by Ingolia for yeast and mammalian cells (McGlincy and Ingolia, 2017).

Ribosome profiling has been used in bacteria to identify which regions of the genome are translated, revealing novel small proteins and internal initiation sites in annotated ORFs (Shell *et al.*, 2015; Meydan *et al.*, 2019; Weaver *et al.*, 2019). Many studies report changes in gene expression that occur upon a

change in the environment, the imposition of an external stress, or the depletion of a regulatory factor (Liu *et al.*, 2013; Subramaniam *et al.*, 2013; Guo *et al.*, 2014; Haft *et al.*, 2014; Zhang *et al.*, 2018). These studies are powerful because in exponentially growing bacteria the level of translation correlates strongly with steady-state protein levels (Li *et al.*, 2014). When supplemented with sequencing of total RNA by conventional RNA-seq, ribosome profiling data provide estimates of translational efficiency (the number of ribosomes per message). These estimates have yielded important conclusions about how the translation of proteins that form a complex occurs in the correct stoichiometry, even from a single mRNA (Li *et al.*, 2014; Lalanne *et al.*, 2018), and identified mRNA structure as one of the primary determinants of translational efficiency (Burkhardt *et al.*, 2017).

In addition to these studies of gene expression, ribosome profiling can also be used to probe features of the translational machinery and the distinct phases of translation. Mechanisms of action of elongation inhibitors can be studied *in vivo* at codon resolution (Kannan *et al.*, 2014; Marks *et al.*, 2016). Factors involved in regulating translation can also be studied in detail using profiling (Balakrishnan *et al.*, 2014; Elgamal *et al.*, 2014; Woolstenhulme *et al.*, 2015; Baggett *et al.*, 2017). Another successful approach has been immunoprecipitation of factors of interest that associate with ribosomes, followed by ribosome profiling to determine where on mRNAs these factors are engaged (Oh *et al.*, 2011). This approach has led to insights into protein folding and complex-formation during translation.

We are primarily interested in using ribosome profiling to measure local elongation rates which vary as a result of ribosome pausing at mRNA structures, rare codons, or troublesome nascent peptide sequences. Sites where ribosomes pause are expected to yield a higher number of reads than sites where elongation is fast, leading to enriched ribosome density. For these studies which rely on high resolution, it is important that the procedure to capture ribosome footprints truly reflects ribosomes in their native environment.

In our efforts to optimize the protocol for bacteria, where no standard protocol has emerged, we identified artifacts that interfere with capturing the native distribution of ribosome footprints (Mohammad *et al.*, 2019). First, ribosome footprints in bacteria vary widely in size from as short as 15 nt to as long as 40 nt. It is essential to capture all of these footprints to have an accurate view of translation. Second, the way that cells are harvested can alter translation and induce artifacts: addition of chloramphenicol before centrifugation induces strong pauses and leads to pile-ups of ribosomes at the 5'-end of ORFs. Harvesting by filtration also induces pauses at specific codons due to rapid depletion of aminoacyl-tRNAs. Third, translation continues after cell lysis, even in the presence of chloramphenicol. To address these problems, we developed a method of directly freezing the culture and immediately inhibiting translation with a high concentration of MgCl₂. These improvements capture ribosomes as they were under growth conditions and will enable studies to more accurately detect ribosome position *in vivo*. For studies primarily concerned with the number of ribosomes per message (and not their exact position) we also provide the earlier filtration protocol which is simpler and useful for preparation of samples for RNA-seq.

Materials and Reagents

1. Whatman cellulose nitrate 0.45 µm filters (VWR, catalog number: 7184-009)
2. Corning® Costar® Spin-X® centrifuge tube filters (Sigma-Aldrich, catalog number: CLS8163-100EA)
3. Corning® Costar Cell Lifter (plastic spatula) (Sigma-Aldrich, catalog number: CLS3008)
4. 3M Overhead Sheets (PP2500)
5. Razor blades
6. Saran wrap
7. 50 ml conical tube
8. Ti 70 polycarbonate tube
9. 18 G needle
10. P1000 tips
11. Aluminum foil
12. CircLigase™ ssDNA Ligase (Epicentre, catalog number: CL4115K)
13. SuperScript® III Reverse Transcriptase (Invitrogen, catalog number: 18080-044)
14. T4 RNA Ligase 2, truncated (NEB, catalog number: M0242L)
15. T4 Polynucleotide Kinase (PNK) (NEB, catalog number: M0201L)
16. Ambion SUPERase·In™ (Invitrogen Life Technologies, catalog number: AM2696)
17. Nuclease S7 Micrococcal nuclease (MNase) (Roche, catalog number: 10107921001)
18. RNase Free DNase I (Roche, catalog number: 04716728001)
19. Phusion® High-Fidelity DNA Polymerase (NEB, catalog number: M0530L)
20. O'RangeRuler 10 bp Ladder (ThermoFisher, catalog number: SM1313)
21. Primers
 - a. Universal miRNA cloning linker (New England BioLabs, catalog number: S1315S)
 - b. Oligonucleotides from IDT:

Size control 15 mer (100 nmol) (5'-AUGUACACGGAGUCG)
Size control 45 mer (100 nmol):
(5'-AUGUACACGGAGUCGACCCGCAACGCGAUGUACACGGAGUCGACC)
 - c. RT primer NI-NI-9 (250 nmol, DNA oligo, 96 bases, PAGE purified Ultramer) (/5Phos/AGATCGGAAGAGCGTCGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC/iSP 18/CACTCA/iSp18/TTCAGACGTGTGCTCTCCGATCTATTGATGGTGCCTACAG-3)
 - d. PCR primer Forward (100 nmol; not gel purified):
(5'-AATGATAACGGCGACCACCGAGATCTACAC)
 - e. PCR primer Reverse (100 nmol; not gel purified): (5'-CAAGCAGAACGGCATACGAGATNNNNNNNTGACTGGAGTCAGACGTGTGCTCTTCCG)
22. Isopropanol

Note: The underlined region of PCR primer Reverse is the sequence that corresponds to the reverse complement of the barcode.

23. Ethanol
24. Dry ice
25. EGTA
26. MilliQ H₂O
27. Sodium acetate
28. PEG 8000
29. Chloroform
30. NaOH
31. MgCl₂
32. NH₄Cl
33. CaCl₂
34. Triton X-100
35. NP-40
36. SDS
37. EDTA
38. DTT
39. NaOAc
40. MOPS EZ Rich Defined Medium (Teknova, catalog number: M2105)
41. Phenol, Saturated pH 4.5 (VWR, catalog number: 97064-714)
42. Oligo Clean and Concentration kit (Zymo, catalog number: D4061)
43. Ribo-Zero™ Magnetic Kit (Gram-Negative Bacteria) (Epicentre, catalog number: MRZGN126)
44. Agilent High Sensitivity DNA Kit (Bio Analyzer) (Agilent Technologies, catalog number: 5067-4626)
45. GlycoBlue (Invitrogen Life Technologies, catalog number: AM9516)
46. SYBR® Gold Nucleic Acid Gel Stain (Invitrogen Life Technologies, catalog number: S-11494)
47. 1 M Tris, pH 8 (Life Technologies, catalog number: AM9856)
48. D-Sucrose (Fisher, catalog number: BP220-1)
49. 15% TBE Urea 1.0 mm (12 + 2) 45 µl gel (Bio-Rad, catalog number: 345-0091) (2-3 per experiment)
50. 10% TBE Urea 1.0 mm (12 + 2) 45 µl gel (Bio-Rad, catalog number: 345-0088) (2-3 per experiment)
51. 10% TBE Urea 1.0 mm (18 + 2) 30 µl gel (Bio-Rad, catalog number: 345-0089) (1 per experiment)
52. 10% TBE Native 1.0 mm (12 + 2) 45 µl gel (Bio-Rad, catalog number: 345-0051) (4-6 per experiment)
53. 10x Lysis Buffer (50 ml) (see Recipes)
54. Pelleting Buffer (see Recipes)
55. Resuspension Buffer (see Recipes)
56. 10% Sucrose Gradient Buffer (see Recipes)

57. 50% Sucrose Gradient Buffer (see Recipes)
58. RNA Elution Buffer (50 ml) (see Recipes)
59. DNA Elution Buffer (50 ml) (see Recipes)

Equipment

1. Kontes 90 mm filtration apparatus
2. Spex Sampleprep 6870 Freezer Mill
3. Beckman Coulter Optima L-80 XP Ultracentrifuge
4. SW41 Ti rotor
5. Type 70 Ti rotor
6. Polyclear Centrifuge tubes (Seton, catalog number: 7030)
7. Polycarbonate bottle with cap for Ti 70
8. Biocomp Gradient Maker and Fractionator
9. Thermocycler
10. Eppendorf Thermomixer R
11. GE Typhoon Imager
12. Agilent 2100 BioAnalyzer

Software

1. Analysis of sequenced ribosome footprints was done using a custom python pipeline found in the link below: https://github.com/greenlabjhmi/2018_Bacterial_Pipeline_riboseq

Procedure

This protocol details every step of ribosome profiling from culturing cells up to PCR amplification of ribosome footprints in preparation for next-generation sequencing (NGS). A general outline of the workflow is described below:

Day 1

- Growth of bacterial cultures
- Harvest by whole culture freeze
- Harvest by filtration (for total RNA extraction for RNA-seq)
- Cell lysis by freezer mill

Day 2

- Pellet ribosomes over sucrose cushion
- Nuclease digestion

- Sucrose gradient and monosome isolation

Day 3

- Phenol chloroform extraction
- Size selection gel

Day 4 (can split into 2 days)

- Gel extraction
- Dephosphorylation
- Linker ligation
- rRNA depletion
- Reverse transcription
- RT gel

Day 5

- RT gel extraction
- Circularization
- Pilot PCR
- Pilot PCR gel
- Preparative PCR
- Preparative PCR gel

Day 6

- Preparative PCR gel extraction
- Sequencing and data analyses

Day 1

A. Growth of bacterial cultures

Bacterial cultures should be grown in the appropriate medium and growth conditions. When preparing cultures, consider the volume necessary to obtain around 1 mg of total RNA. The following conditions are used for *E. coli* during exponential growth:

1. Inoculate an overnight starter culture (25 ml of MOPS EZ Rich media) from a single colony.
2. The next day, prepare 300 ml of MOPS EZ Rich media and warm at 37 °C for 1 h prior to inoculation.
3. Inoculate 300 ml of MOPS media with 1 ml of overnight culture.
4. Grow to an OD₆₀₀ = 0.3-0.5 (~2 h).

B. Harvest by whole culture freeze

This method of harvesting captures ribosomes without inducing artifacts from stress seen from harvesting by filtration or centrifugation. Under the growth conditions above, only 100 ml of the 300 ml will be flash frozen. The rest can be filter harvested to collect RNA for RNA-seq or frozen as backup.

1. While cells are growing, prepare a liquid nitrogen bath:
 - a. Line an ice bucket with 2-3 layers of aluminum foil (see Figure 1A).
 - b. Fill bucket halfway with liquid nitrogen.
2. When cells are at the correct OD₆₀₀, use a 50 ml serological pipet and spray 50 ml of the culture directly into the liquid nitrogen bath. Make sure not to spray in one area. Instead make circles in the N₂ (see Figures 1B and 1C).

A



B



C



Figure 1. Liquid nitrogen bath used to freeze cultures. A. Line a standard lab ice bucket with 2-3 layers of aluminum foil. B. Add 2 inches of liquid nitrogen, then drip 100 ml of the bacterial culture into the liquid nitrogen. C. Drips should form ice pellets about 0.25 to 0.5 inches in diameter.

3. Repeat Step B2 to collect a total of 100 ml of culture.
4. If collecting cells for RNA-seq, immediately run the rest of the culture (200 ml) through the filtration harvest protocol below to collect cell pellets.
5. Lift the aluminum foil and pour off the liquid nitrogen from the sample. Scrape the frozen pellets into several 50 ml conical tubes on dry ice. Avoid thawing.

C. Harvest by filtration (for total RNA extraction for RNA-seq)

Note: This step is needed to create a sample matched RNA-seq library and can be skipped if RNA-seq is not necessary.

1. Set up the Kontes 90 mm filtration apparatus with Whatman cellulose nitrate 0.45 µm filters during cell growth.
2. Pour ~200 ml of culture into the Kontes 90 mm filtration apparatus and turn on the vacuum.
3. While the media is filtering, gently but quickly scrape with a plastic spatula along the surface until you get a good clump of cells. Freeze in liquid nitrogen.

Note: You should be able to do this twice in about 60 s. The sample should never go dry—the dryness and cold that comes with losing all the media stresses the cells.

4. Place pellets in a 50 ml conical tube and store at -80 °C, do not allow any thawing.
5. RNA can be extracted using RNA extraction kits or phenol extraction from the cell pellet.
6. Extracted RNA can then be used to create RNA-seq using commercially available kits.

D. Cell lysis by freezer mill

Lysis is done through mechanical grinding of the frozen culture. The lysis buffer used contains 150 mM MgCl₂ to inhibit translation in the lysate.

1. Pre-Chill Mixer Mill grinding cylinders and 3 x 50 ml conical tubes on dry ice.
2. Measure 50 g of frozen culture, making sure to avoid thawing. Add 5.5 ml of 10x lysis buffer to large grinding cylinder and allow it to freeze.
3. Transfer 50 g frozen culture into the grinding cylinder.
4. Grind using the following cycles: 10 cycles, 10 Hz, 5 min precool, 1 min run, 1 min cool.

Note: The grinding protocol used here was optimized to maximize RNA yield while maintaining polysome integrity visualized by sucrose gradient sedimentation. We recommend revalidating grinding settings if using a different model Mixer Mill or a different organism.

5. Transfer the pulverized cells into the pre-chilled 50 ml conical tubes.
6. Store at -80 °C for later use, or continue.

Day 2

E. Pelleting ribosomes over sucrose cushion

1. Thaw lysate on benchtop until thawed (1-2 h, mixing every 10 min). Place on ice immediately after thawed.
2. Pre-clear lysate by spinning for 10 min at 9,000 x g at 4 °C to remove cellular debris.
3. Prepare two sucrose cushions per sample:
 - a. Transfer 3 ml of pre-chilled pelleting buffer into a Ti 70 polycarbonate tube x 2.
 - b. Layer 22 ml lysate gently on top of cushion x 2.
 - c. Spin at 370,000 x g (60,000 rpm) for 1.5 h at 4 °C in a Ti 70 rotor to pellet ribosomes.
4. Remove supernatant from the ribosome pellet and rinse each pellet with 150 µl of pre-chilled Resuspension Buffer without disturbing pellet.
5. To resuspended pellet:

- a. Add 100 μ l of Resuspension Buffer to each pellet.
- b. Gently vortex for 5 min, making sure the ribosome pellet is fully dissolved.
- c. Combine the 2 tubes for a total of 200 μ l resuspended pellet.
6. Measure A_{260} of a 1:100 dilution using water.
7. Calculate the amount needed for 12.5 AU (roughly 0.5 mg RNA), where AU = 1 ml solution with a given A_{260} .
Note: If sample is less than 12.5 AU, lyse and pellet the other 50 ml of frozen culture. Resuspend with the first 200 μ l collected. As little as 5 AU can be used for library preparation, but this is not recommended.
 - a. $12.5 \text{ AU} / (A_{260} * 100/1,000) = \mu\text{l}$ needed (this takes into account the 100-fold dilution)
 - b. Use the calculation above to calculate the volume needed for 12.5 AU, and add Resuspension Buffer to bring volume to 200 μ l.
8. Freeze at -80 °C to store or continue.

F. Nuclease digestion

Use 12.5 AU of sample in 200 μ l that was pelleted for nuclease digestion.

Note: If sample is less than 12.5 AU, scale the MNase accordingly.

1. Add 6 μ l of SUPERase-In.
2. Add 2 μ l of 375 U/ μ l MNase (total of 750 U).
3. Incubate at 25 °C for 1 h shaking at 1,400 rpm (thermomixer).
4. Add 2 μ l of 0.5 M EGTA pH 8 to quench the reaction (EGTA chelates calcium to quench MNase, a calcium dependent enzyme).
5. Return to ice until next step.

G. Sucrose gradient and monosome isolation

1. Fill SW41 Polyallomer tubes with 6 ml of 50% Sucrose Gradient Buffer and layer on top ~6 ml of 10% Sucrose Gradient Buffer (all the way to the top of the tube).
2. Put on rubber caps carefully to allow any air bubbles to escape through the hole in the cap.
3. Rotate the tubes on the Biocomp gradient maker using the following settings:
Time = 1:48 s
Angle = 81.5°
Speed = 17 rpm
4. Remove rubber caps and 150 μ l off the top of the gradient to make room for the sample.
5. Gently layer 200 μ l of the sample on top of gradient and balance in rotor tubes (sample should be approximately 210 μ l).
6. Spin in ultracentrifuge at 201,000 $\times g$ (35,000 rpm) for 2.5 h at 4 °C using acceleration and deceleration setting of 7.
7. Prepare sucrose gradient fraction collector following the manufacturer's instructions.
8. Set fraction collection so that each fraction has approximately 350 μ l.

9. Fractionate the sucrose gradient. Keep ~3 monosome fractions (~1,050 µl) (Figure 2A).
10. Freeze fractions in liquid nitrogen and store at -80 °C.

Day 3

H. Phenol chloroform extraction

Note: Use screwcap tubes and pipette in the fume hood. Spin steps are done on a tabletop centrifuge at > 10,000 x g at room temperature (RT) unless otherwise specified.

1. Pre-warm 750 µl of phenol, saturated pH 4.5 at 65 °C in thermomixer.
2. Add 57.1 µl of 20% SDS to 1 ml of sample.
3. Add sample to heated phenol.
4. Incubate and shake for 5 min at 65 °C at 1,400 rpm on the thermomixer. Occasionally pull the tubes during this period and vortex on a full speed bench vortexer.
5. Chill samples on ice for 5 min.
6. Spin for 2 min.
7. Keep the **bottom aqueous layer** and add to a tube containing 700 µl of phenol, saturated pH 4.5 at room temperature.

Important: Due to the high concentration of sucrose, there is layer inversion and the aqueous layer is on the bottom.

8. Vortex for 5 min at room temperature.
 9. Spin for 2 min.
 10. Keep the **bottom aqueous layer** and add to 600 µl of chloroform
 11. Vortex for 30 s.
- Note: If the samples become cloudy, warm to ~30 °C. This is likely the SDS falling out of solution.*
12. Spin for 2 min.
 13. Keep the **top aqueous layer** (~1 ml) and place in a 15 ml conical tube.
 14. Add 1 volume (1 ml) of MilliQ H₂O to dilute the sucrose.
 15. Add 3 M of sodium acetate (pH 5.5) to a final concentration of 0.3 M (~200 µl).
 16. Add an equal volume of isopropanol and mix well.
 17. Precipitate the RNA (30 min on dry ice, or overnight -20 °C).
 18. Centrifuge in Eppendorf 5804R at 12,000 x g (~9,000 rpm) for 30 min. Set temperature to 4 °C.
 19. Remove as much liquid as possible without disturbing the pellets.
 20. Air dry samples in the hood.
 21. Resuspend in 40 µl of MilliQ H₂O or 10 mM Tris pH 8 and transfer to a 1.7 ml tube.
 22. Measure A₂₆₀.
 23. You will run approximately 20 µg on the size selection gel.
 24. Store samples at -80 °C.

I. Size selection gel

1. Prepare samples:

20 µg Sample RNA + 2x RNA Loading Dye (NEB)

Ladder (oligos: 15 nt and 45 nt) = Mix 3 µl (10 µM stock) of each marker size + 6 µl 2x RNA Loading Dye per lane. Load 6 µl in one lane (enough for 2 gels)

2. Heat samples and ladder at 80 °C for 2 min, then place on ice.
3. Prepare **15% TBE Urea 1.0 mm (12 + 2) 45 µl gel**.
4. Load samples carefully with at least one empty lane between samples.
5. Run gel(s) until bromophenol blue is ¾ down the gel.
If running at 15 W:
1 gel = 17 min
2 gels = 30 min
6. Remove gels from plates and place in SYBR-gold stain (100 ml of 1x TBE + 10 µl of SYBR-gold) for 5 min.
7. Remove from stain and place on transparency film, then cover with saran wrap.
8. Scan on the GE Typhoon Imager using the fluorescence setting for SYBR Gold. Use a 350-400 PMT.
9. Print the gel as actual size and use printout as a cutting guide to cut sections out of the gel. Cut from the top of the upper marker (45 nt) to bottom of lower marker (15 nt) (Figure 2B).
10. Cut out 15 nt and 45 nt markers and treat as a control sample. This can be used to assess proper ligation and reverse transcription later on.
11. Rescan gel to confirm proper cutting.
12. For elution, crush gel by doing the following:
 - a. Place gel cutout into a 0.5 ml tube with holes poked through the bottom (18 G needle).
 - b. Place the 0.5 ml tube in a 1.7 ml tube to collect the gel.
 - c. Spin for 5 min, gel should extrude from the 0.5 ml tube into the bottom 1.7 ml tube.
 - d. Make sure to collect any remaining gel from the 0.5 ml tube.

Day 4 (can be split into 2 days)

J. Gel extraction

1. Add 500 µl of RNA Elution Buffer to each sample.
2. Add 2.5 µl of SUPERase-Inhibitor to each sample.
3. Shake overnight in a thermomixer, 1,400 rpm at 4 °C.
4. The next day, briefly spin samples.
5. Transfer gel slurry into Spin-X columns (using P1000 tips with the ends cut off).
6. Spin for 3 min at 20,000 x g at room temperature.
7. Put eluate into a new 1.7 ml tube.
8. Add 2 µl of GlycolBlue to each sample.
9. Add 650 µl of isopropanol.
10. Precipitate (40 min–dry ice), spin for 30 min at > 10,000 x g, 4 °C.
11. Remove supernatant, and gently wash with ~100 µl of 80% cold ethanol.

12. Dry pellets by speed vac (10 min).
13. Resuspend pellet in 5 µl of MilliQ H₂O.

K. Dephosphorylation

Reaction Mix (per sample)

1 µl of 10x PNK buffer
1 µl of SUPERase-In
2 µl of MilliQ H₂O

1. Heat samples collected from J13 at 80 °C for 2 min and then place on ice.
2. Add 4 µl of Reaction Mix to each sample.
3. Add 1 µl of PNK to each sample (10K U/µl).
4. Incubate at 37 °C for 1 h.

L. Linker ligation

Reaction Mix (per sample):

1 µl of 10x Ligation Buffer
0.4 µl of 50 µM Universal miRNA cloning linker
7.6 µl of 50% PEG 8000

Note: Ligation buffer and PNK buffer are nearly identical, so half of the required ligation buffer is used.

1. Add 9 µl of Reaction Mix to each sample. Any white precipitate is assumed to be glycogen coming out as a result of the PEG.
2. Add 1 µl of T4 RNA ligase 2 truncated to each sample.
3. Incubate at 37 °C for 3 h.
4. Clean up with oligo and concentration kit and elute in 15 µl of MilliQ H₂O.

M. rRNA depletion

Deplete rRNA for all samples except the 15 nt and 45 nt controls.

1. Follow Ribo-Zero kit instructions for rRNA depletion.
2. Clean up with oligo and concentration kit and elute in 15 µl of MilliQ H₂O.

N. Reverse transcription (RT)

Reaction Mix (per sample):

4 µl of 5x RT buffer
1 µl of 10 mM dNTPs
1 µl of 0.1 M DTT
1 µl of SUPERase-In

1. Use 10.75 µl of sample for RT, save the other 4.25 µl.
2. Add 1.25 µl of RT primer NI-NI-9 (10 µM stock) and heat sample to 80 °C for 2 min, then chill on ice.
3. Add 7 µl of Reaction mix to each sample.
4. Add 1 µl of Superscript III RT to each sample (Invitrogen 56575).
5. Incubate at 48 °C for 30 min.
6. Add 2.2 µl of 1 M NaOH to each sample.
7. Heat to 98 °C for 20 min.
8. Add 28 µl of MilliQ H₂O.
9. Clean up with oligo and concentration kit (Zymo) and elute in 10 µl of MilliQ H₂O.

O. RT Gel

1. Prepare Samples:
10 µl of sample cDNA + 10 µl of 2x RNA Loading Dye
5 µl of 10 bp O'RangeRuler ladder
Note: The sample containing the 15 and 45 nt markers will be used to indicate proper ligation and reverse transcription, and act as size markers for gel excision.
2. Heat to 80 °C for 2 min and then put on ice.
3. Prepare **10% TBE Urea 1.0 mm (18 + 2) 30 µl gel**.
4. Load samples carefully with at least one empty lane between samples.
5. Run gel(s) until xylene cyanol is run off the gel.
If running at 15 W:
1 gel = 40 min
2 gels = 60 min
6. Remove gels from plates and place in SYBR-gold stain (100 ml 1x TBE + 10 µl SYBR-gold) for 5 min.
7. Remove from stain and place on transparency film, then cover with saran wrap.
8. Scan on the GE Typhoon Imager using the fluorescence setting for SYBR Gold. Use a 350-400 PMT.
9. Print the gel as actual size and use printout as a cutting guide to cut sections out of the gel. Cut from the top of the upper marker (45 nt) to bottom of lower marker (15 nt) (Figure 2C).
Important: Avoid excess RT primer band (band below 15 nt marker, 110 nt in size determined by the O'RangeRuler ladder).
10. Rescan to confirm proper cutting.
11. For elution, crush gel by doing the following:
 - a. Place gel cutout into a 0.5 ml tube with holes poked through the bottom (18 G needle).
 - b. Place the 0.5 ml tube in a 1.7 ml tube to collect the gel.
 - c. Spin for 4 min, gel should extrude from the 0.5 ml tube into the bottom 1.7 ml tube.

Day 5

P. RT Gel Extraction

1. Add 500 μ l **DNA Elution Buffer** to each sample.
2. Shake overnight in thermomixer 1,050 rpm at 25 °C (room temperature).
3. The next day, briefly spin samples.
4. Transfer gel slurry into Spin-X columns (using P1000 tips with the ends cut off).
5. Spin for 3 min.
6. Put eluate in new 1.7 ml tube.
7. Add 2 μ l of GlycoBlue to each sample.
8. Add 650 μ l of isopropanol.
9. Precipitate (40 min–dry ice), spin for 30 min at > 10,000 $\times g$, 4 °C.
10. Remove supernatant, and gently wash with ~100 μ l 80% cold ethanol.
11. Dry pellets by speed vac (10 min).
12. Resuspend pellets in 15 μ l of 10 mM Tris pH 8.

Q. Circularization

Reaction mix (per sample):

2 μ l of CircLigase™ buffer
1 μ l of 1 mM ATP (from CircLigase™ Kit)
1 μ l of 50 mM MnCl₂ (from CircLigase™ Kit)

1. Add 4 μ l of Reaction Mix to each sample
2. Add 1 μ l of Circ ligase to each sample.
3. Incubate at 60 °C for 60 min.
4. Incubate at 80 °C for 10 min, then put on ice.

R. Pilot PCR

The goal of the PCR step is to amplify the sample while minimizing PCR amplification bias and over-amplification. To estimate the cycles of PCR needed for proper amplification, several pilot cycles are run to visualize the point at which over-amplification occurs.

Reaction Mix (6 samples)

40 μ l of HF Buffer (F-518)
4 μ l of 10 mM dNTPs
1 μ l of 100 μ M PCR primer Forward
2 μ l of Phusion polymerase (F-530)
133 μ l of MilliQ H₂O

1. Prepare Reaction mix.

2. Combine in a 1.7 ml tube:

27 µl Reaction mix

1.5 µl barcoded PCR primer Reverse (10 µM)

1.5 µl Sample

3. Divide this into 3 x 9 µl aliquots into 0.5 ml tubes.

4. Run on PCR protocol:

Step 1: 98 °C–30 s

Step 2: 98 °C–10 s

Step 3: 65 °C–10 s

Step 4: 72 °C–5 s

Step 5: go to step 2–8 times

Step 6: 4 °C–forever

End

Each sample will have 3 aliquots in the PCR machine. Take one aliquot from each sample out of the PCR machine at the end of 5, 7, and 9 PCR amplification cycles.

Notes:

a. *Pause the machine at 72 °C, wait for 5 s.*

b. *Quickly remove tubes and place directly on ice.*

c. *Quickly close the lid and continue the program.*

5. Each sample will now have 3 PCR products made from 3 different PCR cycle numbers.

6. Proceed to Pilot PCR gel.

S. Pilot PCR gel

1. Prepare Samples:

9 µl of PCR sample + 2 µl of 6x DNA Loading Dye

5 µl of 10 bp O'RangeRuler ladder

2. Prepare **10% TBE Native 1.0 mm (12 + 2) 45 µl gel.**

3. Load samples carefully, grouping each sample together with the cycle numbers in ascending order.

4. Run gel(s) until xylene cyanol is run off the gel.

If running at 15 W:

1 gel = 40 min

2 gels = 60 min

5. Remove gels from plates and place in SYBR-gold stain (100 ml of 1x TBE + 10 µl of SYBR-gold) for 5 min.

6. Remove from stain and place on transparency film, then cover with saran wrap.

7. Scan on the GE typhoon Imager using the fluorescence setting for SYBR Gold. Use a 350-400 PMT (Figure 2D).

8. Determine how many cycles are needed to obtain a good band around 150-190 bp without any larger molecular weight products.

*Note: The ideal number of cycles gives you a nice product band but no trace of higher molecular weight products. Be very careful to avoid this because when you see higher molecular weight products, bias is likely being introduced. After determining the appropriate number of cycles from the Pilot PCR, run the Preparative PCR at **1 fewer cycles** to ensure that no high weight bands (usually a smear) emerge.*

9. Proceed to Preparative PCR.

T. Preparative PCR

Determine the proper cycle count for each sample. Follow the same steps as the pilot PCR, but do not divide the reaction.

1. Combine in 1.7 ml tube:

27 µl of Reaction mix

1.5 µl of barcoded PCR primer (10 µM) (can use either Ni or IDX primers)

1.5 µl of Sample

2. Run PCR using the cycle count determined from the pilot PCR.

U. Preparative PCR gel

1. Prepare Samples:

30 µl of PCR sample + 6 µl of 6x DNA Loading Dye

5 µl of 10 bp O'RangeRuler ladder

2. Prepare **10% TBE Native 1.0 mm (12 + 2) 45 µl gel.**

3. Load samples carefully, leaving one lane empty between samples.

4. Run gel(s) until xylene cyanol is run off the gel.

If running at 15 W:

1 gel = 40 min

2 gels = 60 min

5. Remove gels from plates and place in SYBR-gold stain (100 ml of 1x TBE + 10 µl of SYBR-gold) for 5 min.

6. Remove from stain and place on transparency film, then cover with saran wrap.

7. Scan on the GE Typhoon Imager using the fluorescence setting for SYBR Gold. Use a 350-400 PMT.

8. Print the gel as actual size and use printout as a cutting guide to cut sections out of the gel. Cut from the 150 bp marker to the 200 bp marker (Figure 2E).

Important: Avoid the excess RT primer band (runs at 140 bp mark).

9. Rescan to confirm proper cutting.

10. For elution, crush gel by doing the following:

- a. Place gel cutout into a 0.5 ml tube with holes poked through the bottom (18 G needle).

- b. Place the 0.5 ml tube in a 1.7 ml tube to collect the gel.
- c. Spin for 5 min, gel should extrude from the 0.5 ml tube into the bottom 1.7 ml tube.

Day 6

V. Preparative PCR gel extraction

1. Add 500 μ l of DNA Elution Buffer to each sample.
2. Shake overnight in thermomixer—1,050 rpm at 25 °C (room temperature).
3. The next day, briefly spin samples.
4. Transfer gel slurry into Spin-X columns (using P1000 tips with the ends cut off).
5. Spin for 3 min at 20,000 $\times g$ at RT.
6. Put eluate in new 1.7 ml tube.
7. Add 2 μ l of GlycoBlue to each sample.
8. Add 650 μ l of isopropanol.
9. Precipitate (40 min–dry ice), spin for 30 min at > 10,000 $\times g$, 4 °C.
10. Remove supernatant, and gently wash with ~100 μ l of 80% cold ethanol.
11. Dry pellets by speed vac (10 min).
12. Resuspend pellets in 10 μ l of 10 mM Tris pH 8.
13. Check size and concentration using a BioAnalyzer.

W. Sequencing and data analyses

1. Dilute an aliquot of each library to 2 nM and combine together to multiplex.
2. Submit 10 μ l of 2 nM multiplexed sample for 50 cycles, single-end sequencing on the Illumina HiSeq 2500.
3. Process the sequencing data with custom python 2.7 scripts available here:
https://github.com/greenlabjhmi/2018_Bacterial_Pipeline_riboseq.
4. In broad strokes, the Jupyter notebook Github-Ribo_Density.ipynb.
 - a. Remove the linker CTGTAGGCACCATCAATAGATCGGAAGAGCACACGTCTGAACCTCCA GTCA from the 3'-end of reads using Skewer version 0.2.2.
 - b. Use Bowtie version 0.12.7 to align reads to indices including ladder oligos, tRNA, and rRNA; reads that do not map to these indices are then mapped to the *E. coli* genome (NC_000913.2) allowing two mismatches and requiring unique mapping to a single site.
 - c. Write out ribosome density as a WIG file, as a binary file, and a size-separated dictionary using the 3'-end of reads to assign ribosome position.
5. The Jupyter notebook Github-Ribo-Analysis.ipynb provides further analyses including plots of average ribosome density at start or stop codons, the calculation of pause scores at specific codons, reading frame, and the degree of drop-off across genes (their asymmetry scores).

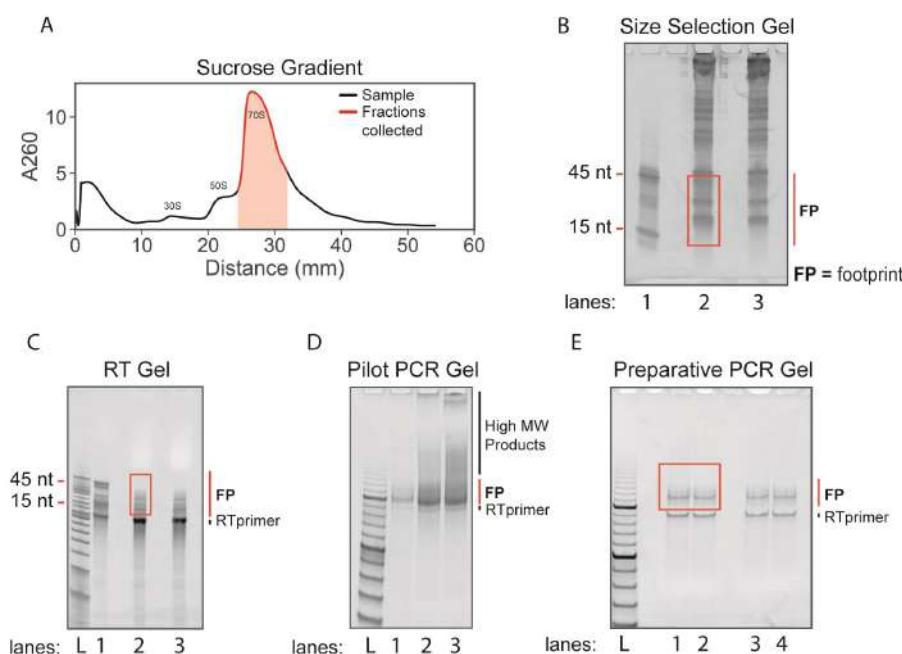


Figure 2. Ribosome profiling sucrose gradients and gels. A. Sucrose gradient trace from Step G9. A volume of ~1,050 μ l was collected (highlighted in red) containing 70S ribosomes. B. Size selection gel from Step I9. Lane 1: 15 nt and 45 nt size markers. Lanes 2 and 3: RNA from fractionated monosomes. The red box indicates the excised region containing ribosome footprints (FP). C. Reverse transcription gel from Step O9. Lane 1: ligated and reverse transcribed 15 nt and 45 nt size markers. Lanes 2 and 3: ligated and reverse transcribed ribosome footprints. The red box indicates excised region which avoids the excess RT primer band. D. Pilot PCR gel from Step S7. Lanes 1-3: PCR amplified products after 5, 7, and 9 PCR cycles. Note the appearance of unwanted high molecular weight products after 7 and 9 PCR cycles. E. Preparative PCR gel from Step U8. The red box indicates excised region which avoids the RT primer band. FP = ribosome footprints. L = O'RangeRuler 10 bp ladder.

Recipes

Note: All concentrations listed are final concentrations.

1. 10x Lysis Buffer (50 ml)

200 mM Tris pH 8

1.5 M MgCl₂

1 M NH₄Cl

50 mM CaCl₂

4% Triton X-100

1% NP-40

MilliQ H₂O

2. Pelleting Buffer

- 1.1 M Sucrose
20 mM Tris pH 8
500 mM NH₄Cl
10 mM MgCl₂
0.5 mM EDTA pH 8
MilliQ H₂O
3. Resuspension Buffer
20 mM Tris pH 8
15 mM MgCl₂
100 mM NH₄Cl
5 mM CaCl₂
MilliQ H₂O
4. 10% Sucrose Gradient Buffer
Each sample uses 6 ml
20 mM Tris pH 8
15 mM MgCl₂
100 mM NH₄Cl
2 mM DTT
10% sucrose
MilliQ H₂O
5. 50% Sucrose Gradient Buffer
Each sample uses 6 ml
20 mM Tris pH 8
15 mM MgCl₂
100 mM NH₄Cl
2 mM DTT
50% sucrose
MilliQ H₂O
6. RNA Elution Buffer (50 ml)
300 mM NaOAc pH 5.5
1 mM EDTA pH 8
MilliQ H₂O
7. DNA Elution Buffer (50 ml)
300 mM NaCl
1 mM EDTA pH 8
10 mM Tris pH 8
MilliQ H₂O

Acknowledgments

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Competing interests

There are no conflicts of interest or competing interest.

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Sulfatase Assay to Determine Influence of Plants on Microbial Activity in Soil

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[Abstract] Sulfatase activity is often used as a measure of the activity of soil microorganisms. It is thus a suitable tool to investigate the response of microbes to plants. Here we present a method to determine the influence of various *Arabidopsis* genotypes on the function of soil microbiota using the sulfatase as a quantitative measure. We grew the plants in soil/sand mix under control conditions and measured the sulfatase activity in soil using a spectrophotometric determination of the product. This protocol can be used to test the contribution of individual genes to control of microbiome assembly through analysis of mutants as well as the influence of environment on plant-microbe interactions.

Keywords: *Arabidopsis*, Plant-microbe interactions, Soil, Microbiome, Organic sulfur, Sulfatase, Bacteria

[Background] Plants in their natural environment interact with plethora of microorganisms, pathogenic as well as beneficial. Many microorganisms are beneficial to plants, e.g., by improving their immunity or their nutrition (Kertesz and Mirleau, 2004; Jacoby et al., 2017; Stringlis et al., 2018). Indeed, the role of bacteria in plant sulfur nutrition has long been recognized (Kertesz and Mirleau, 2004). Sulfur is present in soil mainly bound to organic compounds and thus not available to plants. However, bacteria and fungi can metabolize such organosulfur compounds and release the sulfate group to the rhizosphere, where it can be utilized by plants, and improve so plant sulfur nutrition (Gahan and Schmalenberger, 2014). One of the enzymes catalyzing such reactions is sulfatase. This enzyme, catalyzing the reaction $X-O-SO_3 + H_2O \rightarrow X-OH + HSO_4^-$, is found in many organisms including bacteria, fungi, and humans, but is not present in plants (Gunal et al., 2019). Sulfatase is induced by sulfate limitation and is the basis of plant growth promoting effects of some bacteria (Kertesz and Mirleau, 2004). Sulfatase activity in soil reflects the activity of microbial communities and can be used for estimation of soil health after various treatments (Tejada et al., 2006; Zaborowska et al., 2018) alongside activities of, e.g., β -glucosidase, cellobiohydrolase, chitinase, leucine aminopeptidase phosphatase, and tyrosine aminopeptidase (Maharjan et al., 2017).

However, the sulfatase can be used also as a tool to study plant-microbe interactions. The role of the plant microbiome in improving plant performance and fitness has been increasingly recognized and great progress in the understanding of the assembly of plant microbiome has been achieved (Bulgarelli et al., 2013; Bai et al., 2015). Clearly, plants shape their microbiome composition, even though the mechanisms are largely unknown. However, most microbiome studies are based on DNA sequencing and therefore taxonomic description of the microbiome composition (Jacoby et al., 2017). We have used

sulfatase activity to identify mechanisms by which plants shape their microbiome (Koprivova *et al.*, 2019). Through analyzing the effects of *Arabidopsis* accessions on sulfatase in soil and using the activity for genome-wide association mapping we revealed an important role of the phytoalexin camalexin in the interactions between plant roots and rhizosphere bacteria (Koprivova *et al.*, 2019). The sulfatase assay, which was adapted from (Margesin *et al.*, 2014), proved to be an excellent tool to assess the microbiome activity and the effect of plant genotype on such activity. Therefore, here we present a protocol not only for the core enzymatic activity but also for a full assessment of the effects of *Arabidopsis* genotypes (accessions or mutants) on microbiome function.

Materials and Reagents

1. Tape
2. 0.5 ml Eppendorf tubes
3. Toothpicks
4. 1.5 ml cuvettes
5. Plastic trays, Plant Pots Direct, Heavyweight full seed tray (no holes), catalog number: 2012137PT (Figure 1)
6. Plastic inserts (Plant Pots Direct, Seed Tray Inserts 40, catalog number: 2012111PT) (Figure 1)
7. Plastic Petri dishes (Sarstedt, catalog number: 821.473)
8. Pipette tips (Sarstedt)
9. 2 ml plastic tubes (Sarstedt, catalog number: 72.695.500)
10. Plastic rack for 2 ml tubes, Eppendorf
11. Plastic cuvettes (Brand, catalog number: 759115)
12. Seeds of *Arabidopsis thaliana* (can be obtained, e.g., from NASC *Arabidopsis* Stock Centre, <http://arabidopsis.info/BasicForm>)
13. Sand, Quarzwerke (Frechen, Germany)
14. Soil (e.g., CAS11 soil, Bulgarelli *et al.*, 2012)
15. Murashige Skoog (MS) medium (Duchefa Biochemie, catalog number: MO222.0025), stored at RT
16. Sucrose (Sigma, catalog number: S7903-1KG), stored at RT
17. Potassium-4-nitrophenyl sulfate (Sigma, catalog number: N3877-1G), stored at -20 °C
18. P-nitrophenol (Merck, catalog number: 48549), stored at -20 °C
19. Calcium chloride dihydrate (Sigma, catalog number C3881-1KG), stored at RT
20. Sodium hydroxide (NaOH) (Sigma, catalog number: 71687-500G), stored at RT
21. Sodium acetate trihydrate (Sigma, catalog number: S8625-500G), stored at RT
22. Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) (Merck, catalog number: C1396), stored at RT
23. Potassium nitrate (KNO_3) (Merck catalog number: P8394), stored at RT
24. Potassium dihydrogenphosphate (KH_2PO_4) (Merck, catalog number: 1.04873), stored at RT
25. Ferric EDTA (Fe-EDTA) (Merck, catalogue number: E6760), stored at RT

26. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Merck, catalog number: M2670), stored at RT
27. Liquid nitrogen
28. Glacial acetic acid (Merck, catalog number: A6283), stored at RT
29. Sodium hypochlorite solution ($NaClO$), 12%, Cl (Roth, catalog number: 9062.4)
30. Hydrochloric acid (HCl) 37% (Merck KGaA, catalog number: 1.00317.1000), stored at RT
31. Agarose (Sigma, catalog number: A9539-500G), stored at RT
32. Toluene (Sigma, catalog number: 34866-100 ml), stored under fume hood at RT
33. Modified Long Ashton solution (see Recipes)
34. 0.5 M Acetate buffer (see Recipes)
35. 0.005 M p-nitrophenyl solution (see Recipes)
36. 0.5 M calcium chloride solution (see Recipes)
37. 0.5 M sodium hydroxide solution (see Recipes)
38. Standard p-nitrophenol solution 10 mM (100 ml) (see Recipes)
39. Half strength MS medium with sucrose (1 L) (see Recipes)

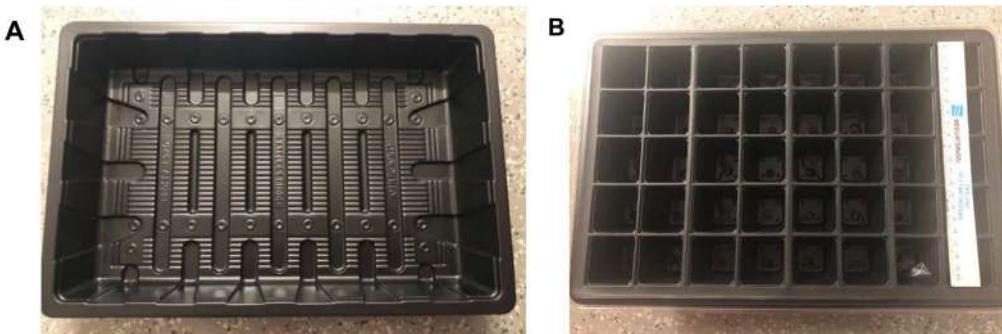


Figure 1. Photos of (A) plastic trays and (B) inserts used for plant growth

Equipment

1. Glass beaker 250 ml
2. Forceps
3. Pipettes (Eppendorf)
4. Balances (Sartorius)
5. Glass beaker
6. Sanyo growth chamber, 10 h light/14 h darkness, 22 °C
7. Rotating shaker, LTF (Labortechnik, Intelli-Mixer, RM-2L)
8. Vortex (LMS, model: VTX-3000L)
9. Incubator for 37 °C (Thermo Scientific, Heratherm Incubator)
10. Centrifuge (Eppendorf, 5424)
11. Spectrophotometer (Eppendorf)

12. Fume hood
13. Computer
14. Desiccator

Procedure

A. *Arabidopsis thaliana* seed sterilization

1. Place small portion of seeds (*ca.* 10 µl) into 0.5 ml Eppendorf tubes.
2. Place open tubes into tube rack inside of desiccator (Figure 2).
3. Add 125 ml of sodium hypochlorite solution into 250 ml glass beaker and place near the seeds.
4. Add 2.5 ml of concentrated HCl into the liquid, which forms chlorine gas, and quickly close the desiccator lid.
5. Sterilize seeds for 3 h.

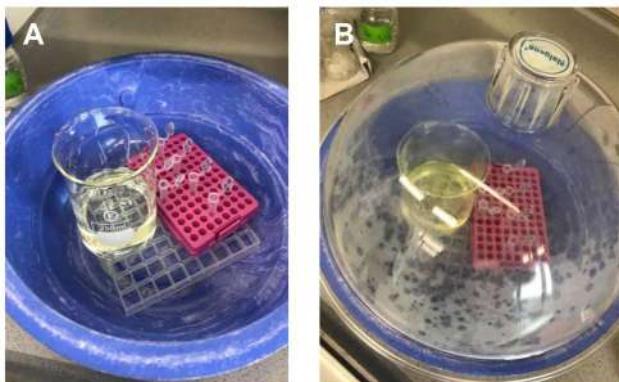


Figure 2. Photos of (A) open or (B) closed desiccator for seed sterilization

B. Initial plates preparation

1. Autoclave half-strength MS medium with 0.8% agarose and 0.5% sucrose and pour it into round Petri dishes, let it set.
2. Carefully place sterile *Arabidopsis* seeds onto agar, using sterile toothpicks, approx. 1 seed per cm².
3. Seal the plates with tape.
4. Place the plates into fridge for 2-3 days for stratification.
5. Place plates into a plant growth cabinet for 9 days (10 h light/14 h dark; 22 °C, 100 µE m⁻² s⁻¹).

C. Preparation of trays

1. Mix soil with sterile (autoclaved) sand 1:9 (V/V).
2. Place plastic insert into the tray.
3. Fill inserts with soil-sand mix.
4. Water slightly

5. Using forceps carefully transfer one seedling per insert, covering roots lightly.
6. Water a bit more, to keep soil moist.
7. Cover tray with the lid.
8. Place trays into plant growth cabinet (10 h light/14 h dark; 22 °C, 100 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 2 weeks, uncover it after 3 days, and water daily with modified Long Ashton solution.

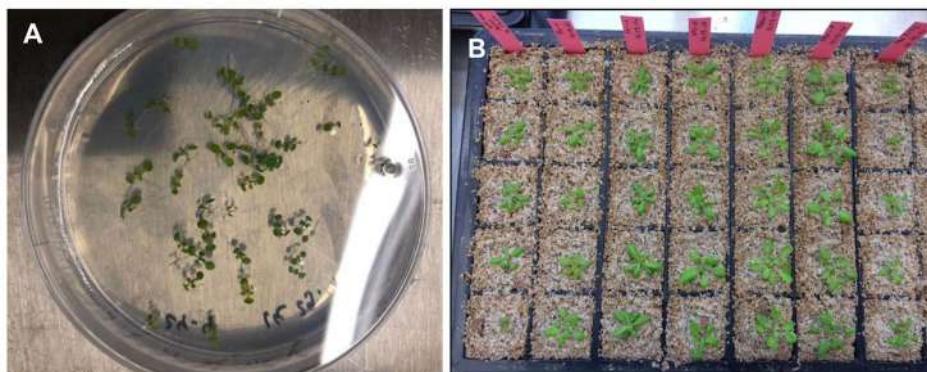


Figure 3. Photos of (A) Petri dish with seedlings before transfer and (B) trays with *Arabidopsis* ecotypes ready for material collection

D. Collection of samples for sulfatase activity

1. Carefully remove plants from soil.
2. Into a 2 ml microcentrifuge tube collect about 1 g of the soil-sand mix, which was closest to the roots of the plant, i.e., rhizosphere, record fresh weight.
3. Collect at least 2 samples per plant.
4. Freeze in liquid nitrogen.

E. Sulfatase activity measurement

1. Defrost the samples in a rack.
2. Add 400 μl of 0.5 M acetate buffer.
3. Vortex each sample for at least 5 s.
4. Under fume hood add 25 μl of toluene.
5. Close tubes and vortex for at least 5 s.
6. Place tubes into rotating rack for 6 min at 100 rpm.
7. Add 100 μl of p-nitrophenyl sulfate solution under fume hood.
8. Vortex each sample for 10 s.
9. Place tubes for additional vigorous shaking for 5 min in an Eppendorf shaker at 1,000 rpm.
10. Place rack with the tubes into 37 °C incubator for 1 h, mixing the whole rack every 10 min by reversing several times.
11. To stop the reaction under fume hood add 100 μl of 0.5 M CaCl_2 solution and 400 μl of 0.5 M sodium hydroxide solution.

12. Vortex each sample for 10 s.
13. Centrifuge tubes at RT at maximum speed for 20 min.
14. Under fume hood carefully transfer the supernatant into plastic 1.5 ml cuvettes.
15. Measure absorption at 400 nm. Use water as blank.
16. Prepare standards by diluting 0, 20, 40, 80, 120, 160, and 200 μ l of 10 mM p-nitrophenol standard to 1 ml H₂O and measure in the same way.
17. Calculate the p-nitrophenol content of the samples from a calibration curve (standards: 0, 200, 400, 800, 1,200, 1,600, and 2,000 nmol ml⁻¹ p-nitrophenol).
18. Using recorded FW data and incubation time of 1 h calculate sulfatase activity in nmol g FW⁻¹ h⁻¹.

Data analysis

The assay determines the end concentration of the sulfatase product, p-nitrophenol, using a calibration curve (Figure 1) and the activity can be calculated from the weight of soil and time of the assay. All calculations can be easily performed in standard office software, e.g., Excel (Table 1). Five biological replicates with two technical replicates each should be used for each plant genotype.

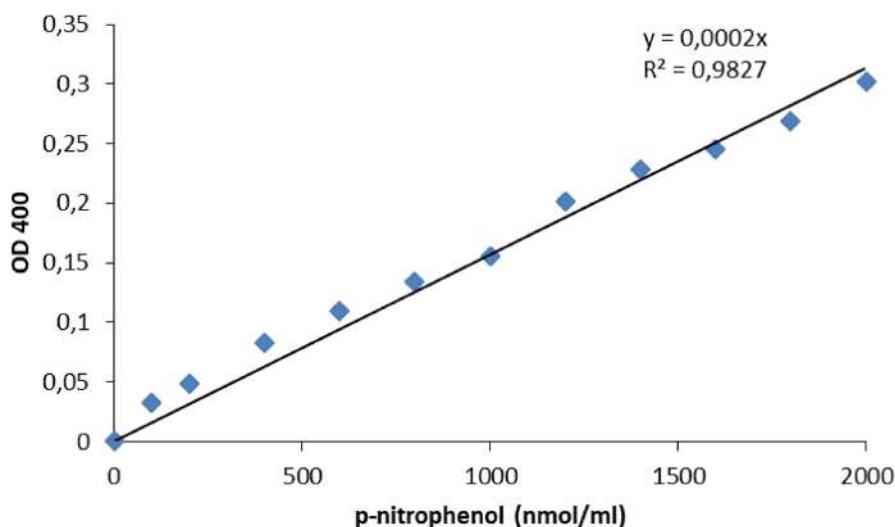


Figure 4. Calibration curve for measurement of sulfatase activity. Standard p-nitrophenol solution was diluted by water to contain 0-2,000 nmol per ml and OD₄₀₀ was measured.

Table 1. Example of calculation of sulfatase activity

sample ID	soil FW ¹ (g)	OD400	p-nitrophenol (nmol)	sulfatase activity (nmol/g FW/h)	Average
Col-0-1	0.1123	0.183	915	8147.8	
Col-0-2	0.1422	0.203	1015	7137.8	
Col-0-3	0.123	0.261	1305	11641.4	
Col-0-4	0.11	0.161	805	6098.5	
Col-0-5	0.111	0.176	880	7926.5	
Col-0-6	0.1121	0.209	1045	9319.5	8409.938
WI-0-1	0.1012	0.1174	587	5800.4	
WI-0-2	0.1211	0.0823	411.5	3398.0	
WI-0-3	0.1113	0.0787	393.5	3535.5	
WI-0-4	0.1241	0.0999	499.5	4025.0	
WI-0-5	0.1117	0.1042	521	4664.3	
WI-0-6	0.1003	0.1092	546	5443.7	4477.805

¹The soil FW is adjusted from the FW of soil/sand sample and the ratio of soil/sand mix, here 10% soil.

Notes

The assay can easily be adapted for different plant species, different sizes of pots, or different soils.

Recipes

1. Modified Long Ashton solution

1.5 mM Ca(NO₃)₂·4H₂O

1 mM KNO₃

0.75 mM KH₂PO₄

0.1 mM Fe-EDTA

0.75 mM MgCl₂·6H₂O

pH 5.7

2. 0.5 M acetate buffer, pH 5.8 (1 L)

64 g sodium acetate trihydrate

1.7 ml glacial acetic acid

Stored at 4 °C

3. 0.005 M p-nitrophenyl sulfate solution (100 ml)

0.1287 g of p-nitrophenyl dissolved in 100 ml of 0.5 M acetate buffer

Stored at 4 °C

4. 0.5 M calcium chloride solution (1 L)

73.5 g CaCl₂·2H₂O dissolved in 1 L H₂O

Stored at RT

5. 0.5 M sodium hydroxide solution (1 L)

20 g NaOH dissolved in 1 L H₂O

Stored at RT

6. Standard p-nitrophenol solution 10 mM (100 ml)

139 mg p-nitrophenol dissolved in 100 ml H₂O

Stored at 4 °C

7. Half strength MS medium with sucrose (1 L)

2.2 g MS medium

5 g sucrose

8 g agarose

pH 5.7 with 1 M KOH

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Transcervical Mouse Infections with *Chlamydia trachomatis* and Determination of Bacterial Burden

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[Abstract] *Chlamydia trachomatis* is an obligate human pathogen. It infects the genital tract of humans ascending into the fallopian tube, exacerbated by chronic pelvic pain, pelvic inflammatory disease, and fallopian tube scarring resulting in infertility and other malignancies. The major hurdle in controlling *chlamydial* spread is that the infection remains asymptomatic, thus leading to chronic, recurrent and persistent infections, with no vaccines developed so far. Being a human pathogen, we do not have an *in vivo* model of *C. trachomatis* infection. *C. trachomatis* do not cause ascending infections and fallopian tube pathology in the mouse urogenital tract when infected vaginally. To overcome this hurdle trans cervical method of infection must be adapted. In this protocol the method of establishing trans-cervical *Chlamydial* infection with the procedure to determine the bacterial load is detailed. This method will facilitate to deliver the bacteria past the cervix establishing an ascending infection into the uterine horns reciprocating human fallopian tube infections.

Keywords: *Chlamydia trachomatis*, Transcervical infection, Bacterial burden, Hydrosalpinx

[Background] Sexually transmitted infections (STI) remain major societal and economic burden despite public health initiatives, vaccinations and development of antibiotics (O'Connell and Ferone, 2016). There is estimated to be 357 million new infections annually and STIs have been identified by the WHO as a global priority for elimination (WHO Global Health Sector Strategy on Sexually Transmitted Infections 2016-2021). Among the various bacterial STI, *Chlamydia trachomatis* is the most common STI (Starnbach, 2018) and stands first with a 131 million new infections annually worldwide (Poston *et al.*, 2017). High reinfection in the genital tract is exacerbated by chronic pelvic pain, pelvic inflammatory disease, and fallopian tube scarring (O'Connell and Ferone, 2016) resulting in infertility and other malignancies. The major hurdle in controlling *chlamydial* spread is that the infection remains asymptomatic, thus leading to chronic, recurrent and persistent infections, with no vaccines developed so far.

Chlamydia undergoes a unique, biphasic developmental cycle that generally modulates between two morphological forms. Extracellular, infectious elementary bodies (EBs) attach to host cells within 15 min of infection, after which they are internalized into a membrane bound vesicle called an inclusion. EBs then differentiate into metabolically active, non-infectious, reticulate bodies (RBs) in 12 h that undergo binary fission, followed by secondary differentiation into EBs after 36 h post infection (hpi). Within/after

48 hpi most of the bacteria is in the EB form and are released upon host cell lysis or extrusion (Abdelrahman and Belland, 2005).

Chlamydia trachomatis being an obligate human pathogen lacks an *in vivo* model. To evaluate specific vaccine antigens that can be used in humans, and to study the host pathogen interaction to understand the pathogenicity of *Chlamydia* infection, a suitable *in vivo* model need to be established (De Clercq *et al.*, 2013). Unlike in *Chlamydia muridarium*, a natural pathogen in mouse, intravaginal inoculation of the bacteria only leads to a mild genital infection and does not cause ascending infection or tubal pathology. The innate immune response in the lower genital tract of the mouse can rapidly eliminate human *chlamydial* organism (Sturdevant and Caldwell, 2014). Thus a higher number of infectious particles are required to establish infection, which yields a 2 log units lower bacterial load in the uterine horns (Darville *et al.*, 1997; Ramsey *et al.*, 2000). To address this limitation an intra-bursal mode of infection was established (Carmichael *et al.*, 2013), via a survival surgery and directly inoculating the bacteria into the bursal region. This method bypasses both the cervical barrier and endometrial lumen, thus do not resemble the natural path of vaginal infection (see Figure 1).

The primary site of *Chlamydia trachomatis* infection is the cervical epithelium (Buckner *et al.*, 2016). Subsequently, the infection ascends to the upper genital tract tissues, the uterine horns and oviducts, which frequently lead to hydrosalpinx, fibrosis, and infertility, which are also common post-infection sequelae in women (Morrison and Caldwell, 2002; Shah *et al.*, 2005). Mice have a bicornuate uterus with two lateral horns and are lined with ligaments carrying blood and lymphatic vessels. The cervix has cranial, fundal and caudal segments. The caudal segment or cervix consists of a single cavity that protrudes into the vaginal opening. To establish an ascending infection, which closely resembles infection in human uterus and fallopian tube, we need to bypass the cervix and inoculate the pathogen into the uterine fundus. This leads to an easy establishment of ascending infection in mouse. This method has been made used in various study (Gondek *et al.*, 2012; Pal *et al.*, 2018; Rajeeve *et al.*, 2018).

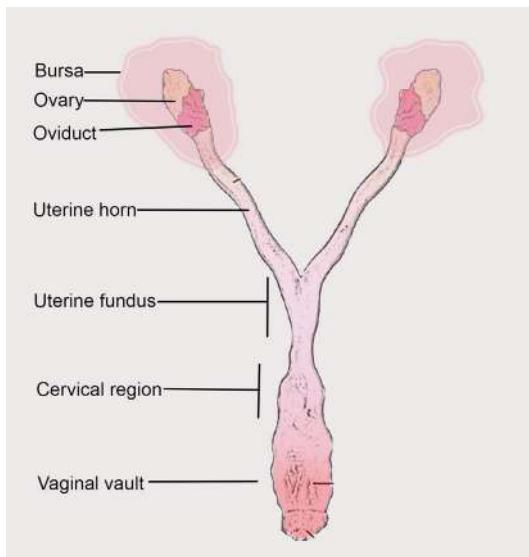


Figure 1. Mouse reproductive tract with parts labelled

Materials and Reagents

Transcervical infection and DNA extraction

1. Eppendorf tubes (Stastedt GmbH, catalog number: 72.688.004)
2. Ultracentrifugation tubes (Beckman Coulter, catalog number: NC9146666)
3. 15 ml Falcons (Corning, catalog number: 430791)
4. 50 ml Falcon
5. 12-well and 24-well plate
6. Rubber scraper
7. 75 cm² or 150 cm² flasks (Corning, catalog numbers: CLS3290 and CLS3291)
8. Nitrile glove (Microflex Neotouch, catalog number: 93-833)
9. Sterile 200 µl pipette tips (Stastedt GmbH, catalog number: 25-201)
10. 1 ml syringe (BD Leur-Lok™, catalog number: 309628)
11. NSET: Non-surgical embryo transfer device (ParaTechs Corp, Product number: 60010)
12. Post-pubertal and sexually mature (> 42 days old) female mice (any strain)
13. Medroxy-progesterone acetate (Depo-provera R 250 mg tablets; Hexal)
14. *Chlamydia trachomatis* L2 stock (1 x 10⁷ bacteria: ATCC VR-902 B)
15. RPMI Media 1640+ GlutaMax (Gibco, catalog number: 11875)
16. Heat inactivated Fetal Calf Serum (FCS) (Sigma, catalog number: F2442)
17. DNeasy blood and tissue kit (Qiagen, catalog number: 69504)
18. Tissue homogenization beads (MP Biomedicals, catalog number: 6913-500)
19. 1x DPBS (Gibco, catalog number: 14190)
20. Sucrose (Sigma, catalog number: S0389)
21. Glutamic acid (Sigma, catalog number: G1251)
22. Nuclease free water (Sigma, catalog number: 7732-18-5)
23. SYBR green (Thermo Scientific, catalog number: A25741)
24. Primers (Sigma)
25. Meglumin diatrizoate (Sigma, CAS: 131-49-7)
26. Sodium diatrizoate hydrate (Sigma, CAS: 737-31-5)
27. Sodium Citrate dehydrate (Sigma, catalog number: 106447)
28. EDTA (Sigma, CAS: 60-00-4)
29. HBSS (Sigma, catalog number: H4385)
30. Renografin
31. 10 mM sodium phosphate (8 mM Na₂HPO₄ and 2 mM NaH₂PO₄; Sigma, CAS: 7558-79-4; CAS: 7558-80-7)
32. SPG buffer (see Recipes)
33. Renografin (see Recipes)

Equipment

1. Pipettes
2. Incubator (ThermoScientific)
3. Centrifuge (Beckman Coulter, model: Avanti™J 25I)
4. Ultra centrifuge (Beckman Coulter, model: Optima L-80 XP)
5. -80 °C freezer (New Brunswick Scientific)
6. RT-PCR instrument (ABI 7500)

Software

1. Microsoft Excel
2. Step One Plus software package (ABI 7500)
3. GraphPad Prism 7

Procedure

A. Preparation of bacterial stock

Note: The protocol below is for Ct grown in two 75 cm² flask of cells.

Preparation of *Chlamydial* stock (Mukhopadhyay *et al.*, 2004; Rajeeve *et al.*, 2018).

1. For infections in mice, *Chlamydia trachomatis* (*Ct*) is propagated in mouse embryonic fibroblast (MEFs). Cells are grown in 75 cm² or 150 cm² flasks in RPMI with 5% heat inactivated FCS.
2. The cells are infected with *Ct* for 48 h. The cells are scraped with a rubber scraper and collected in a sterile 50 ml Falcon.
3. The cells are lysed using glass beads for 3 min by vortexing to release the bacteria.
4. The lysate is pelleted to remove the cell debris by centrifuging at 1,344 x g for 10 min at 4 °C.
5. The lysate from Step A4 is centrifuged at 30,000 x g for 30 min. The pellet is washed with 10 ml 1x SPG buffer at 30,000 g for 30 min.
6. Now the pellet is resuspended in 2 ml of SPG buffer and passed 5 times through 20 G syringe and later 18 G syringe.
7. To prevent any cell debris, the bacterial suspension should be purified using renografin gradient.
8. Prepare a step 20-50% (vol/vol) Renografin gradient in sterile 38.0-ml high-speed centrifuge tubes (Beckman Coulter polycarbonate coated).
9. The gradient should be made with 3.0 ml of 50% Renografin and 7.0 ml of 20% Renografin carefully layered above the 50% Renografin.
10. Add 2.0 ml of the *Ct* suspension onto the top of the 20% phase.
11. Centrifuge at 60,000 x g for 60 min. The EB will appear as a distinct band the 20-50% interface. This layer should be carefully separated and diluted with ice-cold SPG buffer. Repeat a centrifugation step at 30,000 x g and collect the bacterial pellet (it can be very sticky).

12. Resuspended bacterial suspension is made into small aliquots (20-50 μ l) and frozen at -80 °C until used. One aliquot should be used to check the multiplicity of infection (MOI). We need 1×10^6 - 1×10^7 bacteria to infect a mouse.

B. Determine the multiplicity of infection (MOI)

1. Hela cells are plated in duplicate on 12-well plate with glass slides to get a 60-70% confluence next day.
2. Different concentrations of bacterial aliquot in different dilution from 1:10 to 1:10⁻¹⁰ are used to infect each well (e.g., 0.25 μ l, 0.50 μ l, 1 μ l, 2 μ l and so on).
3. After 48 hpi, the MOI is calculated by counting the number of inclusions and number of cells. A MOI of 1 indicates one bacterium infects one cell thereby all cells are infected in a well.

C. Selection of mice

Trans cervical infections are carried out in female mouse, which are sexually matured minimum 8 weeks old. Five-ten female mice should be considered per treatment group. Mice in each experiment should be age-matched and cage mates should be randomly distributed into different treatment groups to avoid cage effect.

D. Transcervical infection

1. To establish a genital tract infection, it is necessary to control the shedding from the uterus. Depo-provera, the hormone progesterone prevents ovulation, thus synchronize the estrus cycle and increase susceptibility to Chlamydia infection. Five days before transcervical infection, mice are injected subcutaneously with 2.5 mg of DepoProvera (medroxy-progesterone acetate): Dissolve one tablet (250 mg) in PBS and use 200 μ l per mouse.
2. On the day of trans cervical infection procedure the bacterial stocks are thawed in ice. One should also carry sterile PBS in 50 ml Falcon, 200 μ l pipette and Non-surgical embryo transfer (NSET) device (Figure 2).

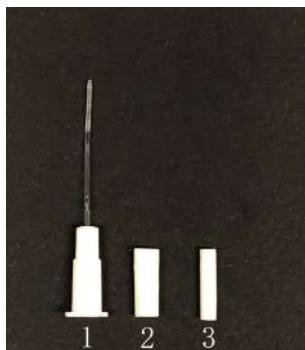


Figure 2. Non-Surgical Embryo Transfer (NSET) device. 1, 2 and 3 indicates the parts of the NSET device.

3. The infection procedure should be performed in a biosafety level 2 facility. The mouse is retrieved from the cage and placed on the top of the grill. Gently hold the tail and allow the mice to grip on the top of the cage. Now orient the mouse so that the posterior part is visible.
4. Gently hold the tail between thumb and forefinger and pull upward that the feet come off the cage. Now use the ring finger to push the back of the mice to angle the hip upwards to easily access the vagina.
5. Carefully insert a 200 μ l pipette tip into the vagina nearly 0.2 cm deep and aspirate liquid that accumulates the cervix. Make sure not to insert too deep that might hurt the mice and lead to internal injury.
6. Now slowly insert the NSET device into the vaginal vault by placing the parts in the order 1, 2 and 3 (Figure 2) as shown in the image and video below (Figure 3) in the order. Pass the needle up and down until it passes through the cervical opening. This needs a few trials and one can use bromophenol blue to confirm passing the cervix (see Video 1).

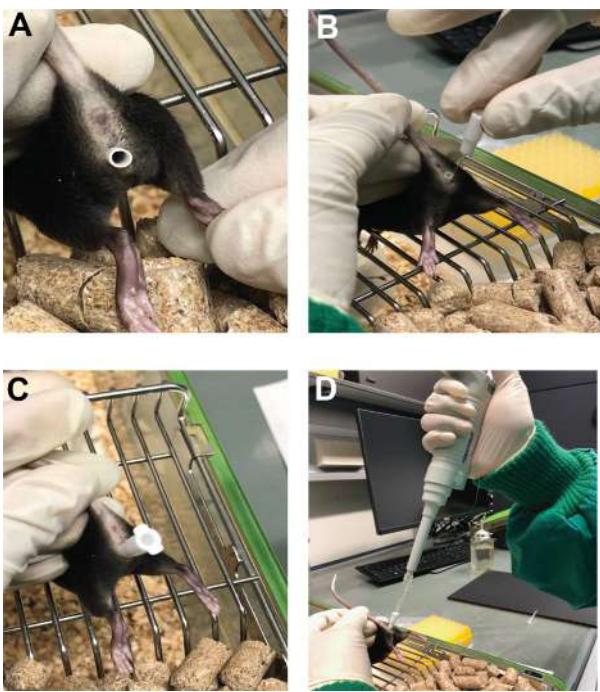


Figure 3. Steps in transcervical infection. A. The mouse is held by the tail as explained above (Step D4) and insert the adaptor of the NSET device into the vagina. B. The NSET needle is further inserted through the adaptor till it reaches the cervix of the mice. C. Make sure that the needle is completely inserted into the cervix. D. One can insert the pipette into the NSET needle and load the bacterial inoculum (refer to the Video 1).



Video 1. Steps in transcervical infection. This video was made at the University of Würzburg under the allowance A2 55.5-2531.01-49/12. The animal experiments were performed in accordance with protocols approved by animal care and experimentation of German Animal Protection Law approved under the Animal (Scientific Procedures) Act 1986 (project license 55.2-2532-2-762).

7. Pipette the required MOI of bacteria through the NSET device and load the bacteria to the uterine fundus. Keep the mouse with the hip upward for 3-5 min to ensure the proper spread of the inoculum. Place the mouse back to its home cage.

E. Bacterial load determination

Copy number detection via RT-PCR

1. The mice were euthanized 7 days post-infection and the uterine horns were taken for further analysis.
2. The uterine horns were homogenized in SPG buffer and DNA was isolated using DNeasy blood and tissue kit (Qiagen) as per the manufacturers' protocol.
3. Quantitative PCR was used to enumerate *Chlamydia* and host genome copy number.
4. To determine the copy number a standard curve was done first with a plasmid encoding the *Chlamydial lytA* gene and mouse gene *Synectin*. One can use any bacterial/host gene for this purpose. The following primers were used for amplifying the *C. trachomatis lytA* gene that was cloned into the vector: fwd, 5'-TCTAAAGCGTCTGGTGAAAGCT-3' and rev, 5'-GAAATAGCGTAGTAATAATACCCG-3'. Normalization of bacterial genome to that of the host was performed using mouse synectin primers: fwd, 5'-ACTAATGTCAAGGAGCTGTACG-3' and rev, 5'-CCTCCGACTTGAACACTTCC-3'.

For creating the standard curve first, the gene of interest (*lyt A* from *Chlamydia/Synectin* from mouse) should be cloned in a suitable plasmid for example TOPO pCR 2.1 vector (Life Technologies, Germany). Different dilutions of the Plasmid are used to perform a standard curve. This gives the primary information on the DNA copy number and the corresponding CT value (Figure 4).

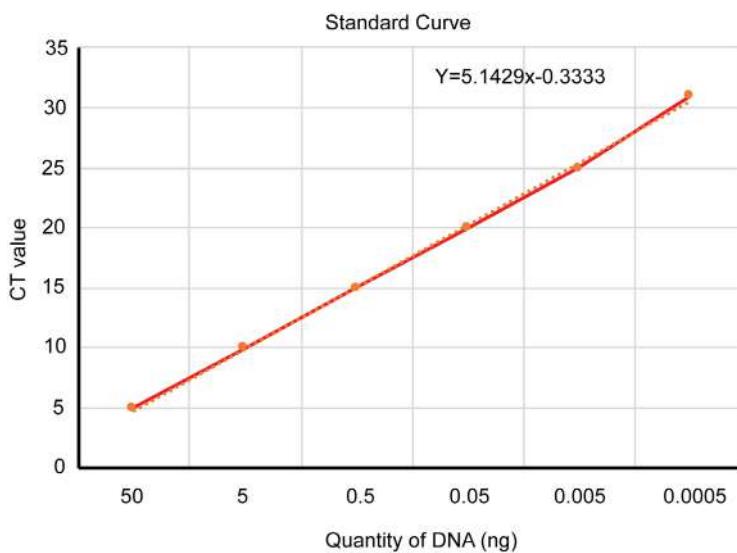


Figure 4. An example of determining the copy number. Different dilutions of plasmid DNA is used to make a standard curve. From the slope of the given line, the amount of DNA present in an unknown sample can be calculated (in this graph the X value) from a corresponding CT value (Y value).

$$\text{The number of DNA copies} = \frac{\text{Amount of the DNA} \times 6.022 \times 10^{23}}{\text{Length (bp)} \times 1 \times 10^9 \times 660}$$

- From the standard curve, the number of copies is determined per μl of the sample as the ratio of *chlamydial* genome to the host genome.

Data analysis

Data were analyzed using Step One Plus software package (Applied Biosystems) and expressed as the ratio of *chlamydial* genome to host genome (*lvtA*/synectin). GraphPad Prism 7 was used to generate a scatter column chart and perform statistical analysis. One-way analysis of variance (ANOVA) with Newman–Keuls multiple-comparison tests was performed with the significance level set to less than 0.01.

Alternate method of Copy number detection:

Since the RT-PCR method can detect DNA from dead bacteria, an alternate method of MOI detection from the mouse can also be used.

- HeLa cells are plated on glass slides in a 24-well plate.
- The uterine horns were either homogenized in SPG buffer or vaginal swabs are collected in SPG buffer.
- The sample is further vortexed with glass beads, and the titers of the chlamydial organisms released into the supernatants are determined on HeLa cell monolayers in duplicate.
- The number of inclusions forming units will give the bacterial load.

5. The total number of IFU per swab are calculated on the basis of dilution factors, inoculation doses, and total sample volumes. An average is taken from the serially diluted and duplicate samples for any given swab. The calculated total number of IFU per swab are converted into \log_{10} , and the \log_{10} IFU counts are used to calculate the mean and standard deviation at each time point.

Notes

1. It is important to have the required MOI in 50-100 μ l of SPG buffer.
2. The bacterial stock that is being used should be tested for mycoplasma contamination.
3. Young mice, which are sexually matured, should be selected for the study. Aged mice do not show a tubal pathology and do not serve as a suitable model.
4. Include 5-10 mice per condition and also a PBS injected mice, a hormone induced heat inactivated *Chlamydia* infected mice as control.
5. When selecting a gene for copy number analysis, one should take care for a single copy gene, if multiple copy is present then the calculations must be adapted accordingly.

Recipes

1. SPG Buffer
10 mM sodium phosphate (8 mM Na_2HPO_4 and 2 mM NaH_2PO_4)
220 mM Sucrose
0.5 mM Glutamic acid, pH 7.4
2. Renografin
26 g Meglumin diatrizoate
4 g Sodium diatrizoate hydrate
0.16 g Sodium Citrate dehydrate
0.02 g EDTA dissolve in 50 ml HBSS, pH 7.4

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Competing interests

The authors declare no competing interest.

Ethics

Statistical analysis was performed to decide the sample size used in mouse infection by the Institute of Mathematics, University of Würzburg under the allowance A2 55.5-2531.01-49/12. All animal experiments were performed in accordance with protocols approved by animal care and experimentation of German Animal Protection Law approved under the Animal (Scientific Procedures) Act 1986 (project license 55.2-2532-2-762).

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Contemporaneous Measurement of Outer and Inner Membrane Permeability in Gram-negative Bacteria

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[Abstract] The emergence and rapid spread of multidrug resistance in bacteria have led to the urgent need for novel antibacterial agents. Membrane permeabilization is the mechanism for many antibacterial molecules that are being developed against gram-negative bacteria. Thus, to determine the efficacy of a potential antibacterial molecule, it is important to assess the change in bacterial membrane permeability after treatment. This study describes the protocol for the assays of outer and inner membrane permeability using the fluorescent probes N-phenyl-1-naphthylamine and propidium iodide. Compared with other experiments, such as electron microscopy and the assay of minimal bactericidal concentration, this methodology provides a simpler, faster, and cost-effective way of estimating the membrane-permeabilizing effect and bactericidal efficacy of antibacterial molecules. This study presents an optimized protocol with respect to the classical protocols by incubating bacteria with antibacterial molecules in the culture condition identical to that of antibacterial assays and then detecting the signal of the fluorescent probe in the buffer without broth and antibacterial molecules. This protocol avoids the effect of nutrient deficiency on the physiological status of bacteria and the interference of antibacterial molecules towards the fluorescent probe. Thus, this method can effectively and precisely evaluate the membrane permeability and match the results obtained from other antibacterial assays, such as minimum inhibitory concentration and time-kill curve assays.

Keywords: Gram-negative bacteria, Antimicrobial peptides, Outer and inner membrane permeability, N-phenyl-1-naphthylamine, Propidium iodide

[Background] Multidrug resistance in bacteria is a major public health crisis. Gram-negative bacteria currently pose the greatest threat to public health because of the emergence and rapid spread of carbapenem resistance. Consequently, new antibacterial molecules must be identified to combat this urgent problem. The outer membrane (OM) of gram-negative bacteria is not only the target of several traditional antibiotics to exert antibacterial activities but also of novel therapeutic agents, such as antimicrobial peptides (AMPs). These molecules can disrupt the OM integrity and cause bacterial lysis through permeabilization. Thus, detecting the permeability of the outer and inner membranes is a direct and important means of assessing the efficacy of antibacterial molecules. Electron microscopy is usually applied to observe the morphological changes in OM, and the assay of minimal bactericidal

concentration is used to examine bacterial viability after treatment. However, these methods are time-consuming and cannot reflect the real-time change in membrane permeability. In this protocol, we provide a convenient and fast approach for evaluating the antibacterial efficacy of agents with potential membrane-permeabilizing effect by using the fluorescent probes N-phenyl-1-naphthylamine (NPN) and propidium iodide (PI).

NPN is a hydrophobic dye that dissolves sparingly in water with very low fluorescent emission. However, the fluorescence intensity increases sharply when NPN binds with nonpolar substances. The intact OM efficiently blocks NPN out of bacteria to ensure that NPN cannot bind to the hydrophobic tail of phospholipids. By contrast, a strong fluorescence emission can be detected when an OM rupture occurs. Thus, the change in fluorescence intensity of NPN can reflect the efficacy of antibacterial molecules on increasing the permeability of OM. PI is a red-fluorescent nucleic acid stain that can bind to DNA and RNA between the bases. Binding to DNA and RNA leads to an enhancement of PI fluorescence by 20- to 30-fold compared with that in aqueous solutions. Given that PI is a membrane-impermeant stain, it can only label bacteria with a compromised inner membrane (IM). Thus, PI is used in this protocol to determine the change in IM permeability after the treatment of antibacterial molecules.

In most of the related studies, the bacterial OM/IM permeability assay is conducted in a 96-well optical-bottom black plate. Bacteria, fluorescent probe, and antibacterial molecules are mixed together and co-cultured throughout the assay, during which the fluorescence intensity is detected at each time point. To avoid the high background fluorescence emitted by the bacterial culture media, the assay buffer (5 mM HEPES, pH 7.2) is used instead. In this case, bacteria grow under an innutritious condition compared with that of the antibacterial assays such as minimum inhibitory concentration and time–kill curve assays, which may affect the physiological status of bacteria. And some antibacterial molecules may also interfere with the function and performance of the fluorescent probe. As a result, the assay may not exactly reflect the change in permeability caused by the antibacterial molecules. The current work presents an optimized procedure with respect to the previously reported protocols (Ma *et al.*, 2016b; Yarlagadda *et al.*, 2016; Krishnamurthy *et al.*, 2019) by culturing bacteria and exposing them to antibacterial molecules in the same medium as those of other antibacterial assays, harvesting bacteria, and detecting the fluorescent yield in the assay buffer at each time point. This methodology can evaluate the membrane permeability more efficiently and precisely and better match the results obtained from the antibacterial assays.

Materials and Reagents

1. Pipette tips
2. 1.5 ml Eppendorf tube (Eppendorf Safe-Lock, catalog number: 022363204)
3. Steritop 0.22 µm filter unit (Millipore Millex-GP, catalog number: SLGP033RB)
4. Flat-bottomed polystyrene 96-well cell culture plates, 0.2 ml well-volume (Corning, Costar®, catalog number: 3599)
5. Glass culture tube 20 mm x 150 mm (sterilized by autoclaving)

6. 50 ml glass conical flask (sterilized by autoclaving) (ShuNiu, China)

7. *E. coli* XJ141026 (Isolated from Xijing Hospital)

Note: Other types of E. coli can also be used.

8. Luria–Bertani (LB) broth (BD/Difco, catalog number: 244620)

9. N-Phenyl-1-naphthylamine (NPN) (Sigma-Aldrich, catalog number: 104043)

10. Propidium iodide (PI (Sigma-Aldrich, catalog number: P4170)

11. Thanatin (synthesized and purified to over 98%)

Note: Thanatin was synthesized with the solid-phase method by applying the Fmoc (9-fluorenylmethyloxycarbonyl) active ester chemistry of as described previously (Fehlbaum et al., 1996). Other AMPs of the experimenter's choice could also be used instead of thanatin.

12. Glucose (Sigma-Aldrich, catalog number: G8270)

13. 1 M HEPES solution (Sigma-Aldrich, catalog number: H0887)

14. 10x PBS stock (Life Technologies, Gibco®, catalog number: 70011-044)

15. Acetone (Sigma-Aldrich, catalog number: 650501)

16. Milli-Q filtered water (ddH₂O)

17. 5 mM HEPES and 5 mM glucose buffer (pH = 7.2) (see Recipes)

Equipment

1. Pipettes

2. Shaking incubator (Zhicheng, model: ZHWY-200D)

3. Static incubators (Taisite Instrument, model: DH4000BII)

4. Microplate spectrophotometer (BioTek, model: PowerWave HT)

5. Tabletop centrifuge machine (Hanil Science Industrial, model: Smart R17)

6. Fluorescence spectrophotometer (Hitachi, model: F-2500)

7. Water purification system (Millipore, model: Milli-Q Advantage A10)

8. Quartz cuvettes with 1 cm path length (Starna Cells, catalog number: 3-Q-10)

Software

1. Gen 5 (Bioteck, USA)

2. FL Solutions 2.0 (Hitachi, Japan)

3. Excel 2016 (Microsoft, USA)

4. Prism 8.0 (GraphPad, USA)

Procedure

A. Sample preparation for the membrane permeability assay

1. Inoculate a single colony of *E. coli* XJ141026 into 5 ml LB broth in a glass culture tube and incubate it overnight in a shaking incubator at 220 rpm at 37 °C.
2. Take 0.1 ml of stationary growth phase of the *E. coli* XJ141026 culture and inoculate 10 ml of the LB broth in a 50 ml glass conical flask. Allow the culture to grow for 12 h at 37 °C and 220 rpm.
3. Add 100 µl of LB broth to 8 wells of the 96-well microtiter plate. Transfer 100 µl of the culture to the first well and make serial twofold dilutions in the LB broth (final volume of 100 µl per well). Determine the OD₆₀₀ of each well via the microplate spectrophotometer.
4. Calculate the dilution factor according to the above serial OD₆₀₀ values. Dilute a portion of the remaining culture to OD₆₀₀ = 0.1 (determined by using the microplate spectrophotometer with a sample volume of 100 µl) in the LB broth to obtain 40 ml diluted bacterial suspension.
5. Transfer the diluted bacterial suspension into two 50 ml glass conical flasks, 15ml per group.
6. Add 12 µl of 1,000 µM thanatin (dissolved in sterile PBS) to one of the 15 ml bacterial suspensions, in which the final concentration of thanatin is 0.8 µM. Add an equal volume of sterile PBS (pH = 7.2) to the other flask and use as the untreated control. Mix well and grow at 37 °C and 220 rpm.
7. Collect the samples of thanatin-treated and untreated cultures at the different time intervals of 0, 30, 60, 120, 180, and 240 min. For each group, 2 ml culture was collected and separately added into two 1.5 ml Eppendorf tubes, 1 ml per tube.
8. Pellet the cells at 8,935 × g for 10 min in 1.5 ml Eppendorf tubes, and remove the supernatant.
9. Wash the cells through resuspension in 1 ml of 5 mM HEPES and 5 mM glucose buffer (pH = 7.2) and the pellet cells via centrifugation at 8,935 × g for 5 min. Remove the supernatant.
10. Repeat Step A9 twice.
11. Resuspend the cells in 1 ml of 5 mM HEPES and 5 mM glucose buffer (pH = 7.2).

B. Outer membrane permeability

1. To determine the outer membrane permeability of thanatin, add 8 µl of 500 µM NPN (dissolved in acetone) to 1 ml resuspended cells (A11) and vortex well.
2. Incubate in darkness for 30 min at room temperature.
3. Turn on the fluorescence spectrometer, and allow the lamp to warm up for 30 min.
4. Define the operating parameters of the spectrometer as follows: photomultiplier tube (PMT) voltage: 700 V, excitation wavelength: 350 nm, and emission wavelength: 420 nm.

Note: The PMT voltage settings are machine- and sample-dependent. To prevent the fluorescence signal values from exceeding the measurement range, the appropriate PMT voltage for the experiments must be established before a series of experiments is started. To set the appropriate PMT voltage, collect and stain the antimicrobial agent-treated bacteria at the

last time point of the experiment, detect the fluorescence signal using different PMT voltages, and choose the optimal PMT voltage that can maximally amplify the signal in the detector range.

5. Carefully clean a fluorescence cuvette with water and ethanol.
6. Transfer 1 ml of the incubated culture to the cuvette and place it in the spectrometer.
7. Start the data acquisition and record.

C. Inner membrane permeability

1. To determine the inner membrane permeability of thanatin, add 5 μ l of 1 mM PI (dissolved in sterile ddH₂O) to 1 ml of resuspended cells (A11).
2. Incubate in darkness for 30 min at room temperature.
3. Define the operating parameters of the spectrometer as follows: PMT voltage: 700 V, excitation wavelength: 535 nm, emission wavelength: 617 nm.
4. Carefully clean the fluorescence cuvette with water and ethanol.
5. Transfer 1 ml of the incubated culture to the cuvette and place it in the spectrometer.
6. Start the data acquisition and record.

Data analysis

1. Repeat the experiment three times independently.
2. Import all the detected values into the GraphPad Prism 8.0 to visualize them graphically. As shown in Figure 1, time and relative fluorescence intensity are depicted in the x- and y-axes, and grouped comparison of untreated control vs. thanatin-treated.
3. Use a two-way ANOVA to analyze the data between the different groups.
4. OM integrity is damaged by Thanatin in a time-dependent manner, and a distinct increase in the fluorescence intensity of NPN is observed 1 h post-incubation (Figure 1A).
5. The increase in fluorescence intensity of PI approximately 2 h post-incubation indicates the increase in IM permeability of the bacteria (Figure 1B).
6. The basal fluorescence (Control group in Figure 1) also increases with time, which may due to the increasing number of aging and dead bacteria along with culture time. Thus, the final fluorescence data in the thanatin-treated group are normalized by subtracting the fluorescence values of the corresponding control group at each time point (Figure 2).

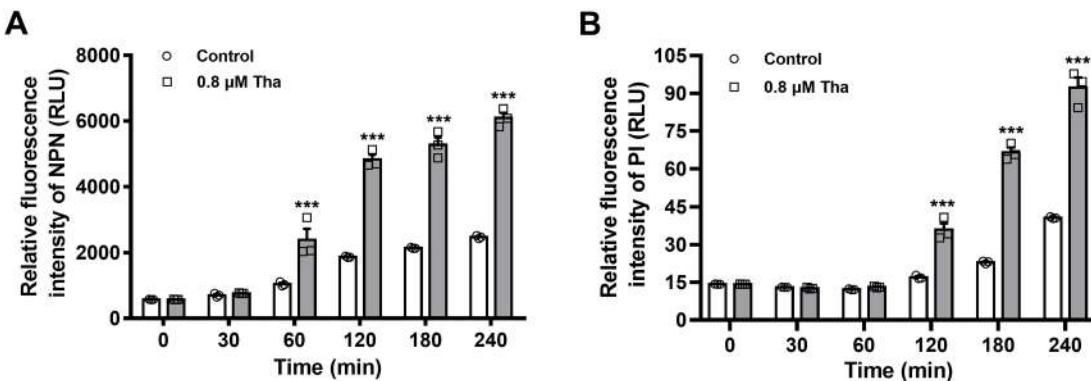


Figure 1. Outer and inner membrane permeabilization of thanatin (Tha) is measured by detecting the fluorescence intensity of NPN (A) and PI (B). All the data are shown as the mean \pm SEM of the three independent experiments. P -values were determined via two-way ANOVA; *** P < 0.001 vs. untreated control (Ma et al., 2019).

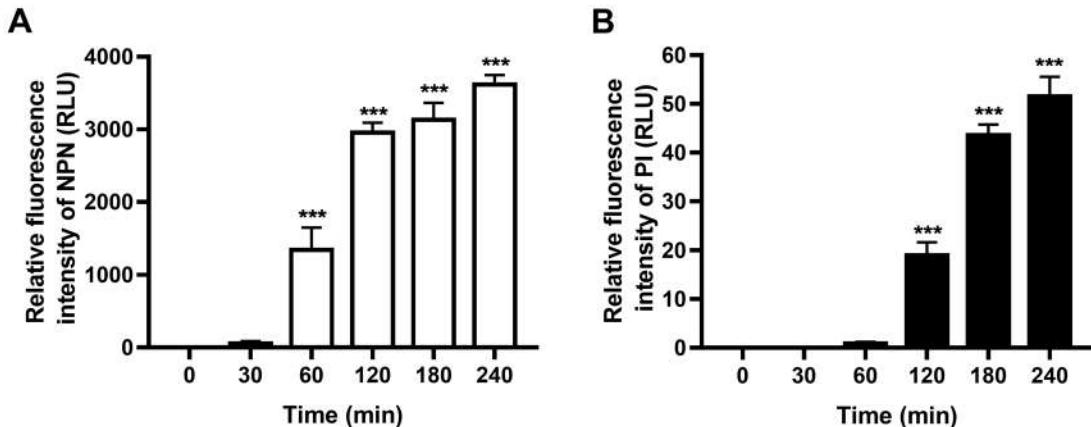


Figure 2. Outer and inner membrane permeabilization of thanatin is measured by detecting the fluorescence intensity of NPN (A) and PI (B) after normalization. All the data are shown as the mean \pm SEM of the three independent experiments. P -values were determined via one-way ANOVA; *** P < 0.001.

Recipes

1. 5 mM HEPES and 5 mM glucose buffer (pH = 7.2)
1 M HEPES solution is diluted to 5 mM HEPES with ddH₂O (pH = 7.2)
Dissolve 90.08 mg glucose in 100 ml of 5 mM HEPES (pH = 7.2)
Sterilize using a 0.22 μ m filter

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This protocol was adapted from previous work (Ma et al., 2016a; Ma et al., 2019). We thank Xiuli Xu and Shan Zhou for providing us with clinical strain from the Department of Clinical Laboratory. Copyright © 2020 The Authors; exclusive licensee Bio-protocol LLC.

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Competing interests

The authors declare no conflict of interest.

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Quantification of Bacteria Residing in *Caenorhabditis elegans* Intestine

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[Abstract] Quantification of intestinal colonization by pathogenic or commensal bacteria constitute a critical part of the analysis to understand host-microbe interactions during different time points of their interplay. Here we detail a method to isolate non-pathogenic and pathogenic bacteria from *C. elegans* intestines, and classify gut phenotypes induced by bacterial pathogens using fluorescently-tagged bacteria. Furthermore, these methods can be used to isolate and identify new culturable bacterial species from natural microbiomes of wild nematodes.

Keywords: Host-Pathogen interactions, *Caenorhabditis elegans*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Escherichia coli*, Colony forming units, Intestinal infection

[Background] In the wild, nematodes are exposed to a wide variety of bacterial and fungal communities (Frézal and Félix, 2015). Under laboratory conditions, the nematode *C. elegans* has been historically maintained in a single food source (Brenner, 1974). However, the worm has been challenged with various pathogens and an increasing number of non-pathogenic-bacteria of diverse nutritive quality (Garsin *et al.*, 2003; Gracida and Eckmann, 2013; Dirksen *et al.*, 2016; MacNeil *et al.*, 2013; Tan *et al.*, 1999). *C. elegans* embryos can be extracted from gravid hermaphrodites by using hypochlorite treatment. This procedure eliminates bacteria allowing the new generation to be exposed anew to a microbe. This advantage provides a unique framework to study host-microbe interactions. Genetic tractability of nematodes and bacteria allows to study eukaryotic (Garsin *et al.*, 2003) and prokaryotic (Gallagher and Manoil, 2001) gene function at different time points on this dynamic interplay.

The response of *C. elegans* to different bacteria depends on both host defenses and bacterial virulence mechanisms (Casadevall and Pirofski, 2003; Hughes and Sperandio, 2008; Casadevall, 2017). Quantifiable physiological outputs (MacNeil *et al.*, 2013; Samuel *et al.*, 2016) and behavioral responses (Zhang *et al.*, 2005; Jin *et al.*, 2016; Palominos *et al.*, 2017) to a variety of microbes have been reported. An important component of the study of bacteria-worm interaction is the quantification of the intestinal bacterial load as well as the ability of bacteria to colonize the animal's gut. Rodriguez Ayala *et al.* (2017) explains how to isolate vegetative and resistant spores of *Bacillus subtilis* from worm's guts. Palominos *et al.* (2017) described two independent methods to accurately quantify bacterial colonization proficiency in *C. elegans*. Here we expand on the methodology used in the latter.

In this protocol we first describe a method to isolate bacteria from guts of worms grown on non-pathogenic (*E. coli* OP50 or OP50-GFP) and pathogenic (*Salmonella* Typhimurium 14028, MST1 or MST1-GFP) bacteria. We then quantify the colony forming units (CFU) as measure of individual colony number present in the intestines of animals. Second, green fluorescent protein (GFP)-expressing

bacteria are used to qualify the degree of intestinal colonization. All of these constitute reliable methods to measure presence of intact bacteria and degrees of colonization of *C. elegans* intestine. Moreover, this protocol is a simple method to measure colonization by any bacteria that is culturable or tagged with fluorescent markers (e.g., beneficial or natural bacterial cohabitants of wild nematodes). This may lead to proper identification of food sources to grow other non-cultured nematodes, as well as to study in a Petri dish how nematodes relates to their natural commensals. Finally, these methods constitute a way to study *in-vivo* interactions between bacteria and its living host for several generations.

Materials and Reagents

1. Laboratory Labeling Tape
2. Disposable Kontes® Pellet Pestle® Grinders (VWR, catalog number: KT749520-0000)
3. Corning® 0.2 µm syringe filters (Sigma-Aldrich, catalog number: CLS431215)
4. 15 ml Falcon tubes (Fisher, catalog number: 14-959-49B)
5. 50 ml Falcon tubes (Fisher, catalog number: 14-432-22)
6. 1.5 ml microcentrifuge tubes (Fisherbrand™, catalog number: 05-408-129)
7. Weighing boats (Fisherbrand™, catalog number: 08-732-112)
8. Pipet tips (Fisher, catalog numbers: 02-707-401, 02-707-415, 02-707-436)
9. 90-mm Petri dishes (Nunc®, catalog number: Z717223-320EA)
10. Microscope slides (Fisher, catalog number: 12-518-100B)
11. 22 x 22 Microscope slide coverslips (VWR, catalog number: 470145-876)
12. Pasteur glass pipette (Fisher Scientific, catalog number: 13-678-20A)
13. Plastic disposable graduated pipettes (Fisherbrand™, catalog number: 13-711-9BM)
14. Borosilicate glass disposable rimless culture tubes (Thomas Scientific, catalog number: 99445-10)
15. *C. elegans* strains; for wild type use Bristol N2 strain from *Caenorhabditis* Genetics Center (CGC)
16. *E. coli* OP50 and OP50-GFP can be obtained from the CGC (<https://cgc.umn.edu/>)
17. *Salmonella* MST1-GFP is available upon request
18. Glycerol ≥ 99.5% (Sigma-Aldrich, catalog number: G9012)
19. NaCl (Merckmillipore, catalog number 106404)
20. BD Bacto™ Peptone (Fisher, catalog number: S71604)
21. Gibco™ Bacto™ Tryptone (Fisher, catalog number: DF0123-17-3)
22. Streptomycin Sulfate (Thermo Fisher Scientific, catalog number: 11860038)
23. Gentamicin sulfate (Sigma-Aldrich, catalog number: G4918)
24. Ampicillin anhydrous basis (Sigma-Aldrich, catalog number: A9393)
25. BD Bacto™ Agar (VWR, catalog number: 90000-760)
26. CaCl₂·2H₂O (Sigma-Aldrich, catalog number: C7902)
27. Cholesterol (Sigma-Aldrich, catalog number: C75209)
28. Ethyl alcohol pure ≥ 99.5% (Sigma-Aldrich, catalog number: 459836)

29. Levamisole hydrochloride ≥ 99% (Sigma-Aldrich, catalog number: 1359302)
30. K₂HPO₄ (Sigma-Aldrich, catalog number: RES20765)
31. KH₂PO₄ (Sigma-Aldrich, catalog number: 1551139)
32. MgSO₄ (Sigma-Aldrich, catalog number: 230391)
33. Yeast extract (Gibco™, catalog number: 212710)
34. Nematode Growth Medium (NGM) plates (see Recipes)
35. Solid Luria-Bertani (LB) (see Recipes)
36. Liquid LB (see Recipes)
37. Sterile 1 M MgSO₄ solution (see Recipes)
38. Sterile 1 M CaCl₂ (see Recipes)
39. Sterile Phosphate buffer (see Recipes)
40. M9 buffer (see Recipes)
41. Levamisole 250 mM (stock) in M9 (see Recipes)
42. M9 + Lev (M9 with 25 mM levamisole) (see Recipes)
43. M9 + Lev + Ab (M9 with 25 mM levamisole with antibiotics) (see Recipes)
44. 1 mM levamisole for microscopy (see Recipes)
45. 87% glycerol for bacterial stocks (see Recipes)
46. 2% agar for microscopy (see Recipes)

Equipment

1. Hand Tally Counter (Humboldt, catalog number: H-9700)
2. Pipetman P10, P200 P1000 (Gilson, catalog numbers: F144802, F123601, F123602)
3. Platinum pick made as described in Wollenberg *et al.* (2013)
4. Bunsen burner
5. Autoclave
6. Stirring hotplate
7. Thermal block
8. Refrigerated Centrifuge (Eppendorf)
9. Laminar flow cabinet
10. Incubator for stable temperature
11. Incubator for liquid culture
12. Dissecting stereoscope with fluorescence
13. Freezer (-20 °C)
14. 500 ml glass beaker
15. 0.5 and 1 L DURAN® Original glass bottles (www.duran-bottle-system.com)
16. Stirrer
17. Inverted fluorescence microscope with x40 and x60 magnification, and Nomarski filters (Nikon, model: Eclipse Ti-5)

Software

1. FIJI (Schindelin *et al.*, 2012) (FIJI is just ImageJ) Version 2.0, available at [http://imagej.net\(Fiji/Downloads\)](http://imagej.net/Fiji/Downloads)
2. Microsoft® Excel 2015
3. GraphPad Prism 6 (©Graphpad Software)

Procedure

Note: All microbiological techniques should be carried out in a clean bench, next to a Bunsen burner.

A. Bacteria and nematode growth

1. Bacteria were stored in 50% glycerol stocks at -20 °C. Stocks were prepared by mixing 287 µl of sterile 87% glycerol and 213 µl of cultured bacteria. Mix by inversion.
2. Streak *E. coli* OP50-GFP and *Salmonella* Typhimurium MST1-GFP from glycerol stocks onto individual Luria-Bertani (LB) plates and grow them overnight at 37 °C. LB plates for these two bacteria should contain ampicillin (50 µg/ml).
3. Next day, pick a single colony and grow it on 10 ml of liquid LB supplemented with 50 µg/ml ampicillin at 37 °C for 6 h on a 50 ml Falcon tube. The OD₆₀₀ should range between 1.5 and 2. Colonies can be picked by using a sterile 200 µl pipette tip, or a smear loop (prior sterilization with fire). Tube lid should be with loose, but secured by lab tape. Shake at 200 rpm.
4. Seed 3 ml of each bacterial culture onto 90-mm NGM agar plates. Allow it to dry and use them next day. Keep at room temperature.
5. Pick 5 L4 worms (genetic background of interest) onto each plate seeded with *E. coli* OP50-GFP and *S. Typhimurium* MST1-GFP. Experiment should be carried out in triplicates.

Allow worms to grow until desired developmental stage (Figure 1). In our case, we allow worms to grow for 2 days and we selected L4 grown on each bacterial lawn, because it is easy to recognize as a specific developmental stage.

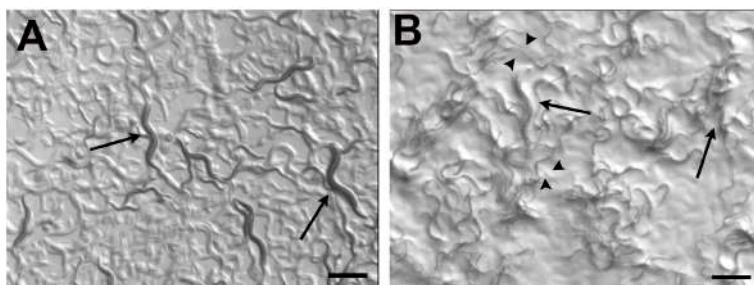


Figure 1. Wild type (N2) worms growing on *E. coli* OP50 and *Salmonella* Typhimurium MST1. As previously described (Palominos *et al.*, 2017), the exposure to bacterial pathogens for two consecutive generations cause that a percentage of *C. elegans* population enter diapause, forming the dauer larvae (arrowheads). Adults can be seen in both conditions (arrows). Scale bars = 500 µm.

B. Isolate bacteria from *C. elegans* intestines: Homogenization and serial dilution

Note: This procedure should be carried out in a sterile area. Ideally, under a laminar flow cabinet. All solutions should be autoclaved prior use.

1. Prepare levamisole (25 mM) in M9 solution (M9 + Lev). This will cause worms to paralyze and stop the pharyngeal pumping, avoiding the solution to enter to the worm's interior. Keep on ice.
2. Select 30 worms of the desired stage grown on each bacterium lawn and pass them with a platinum pick onto 1.5 ml microcentrifuge tubes containing 1 ml of M 9+ Lev. Three tubes should be prepared per bacteria with worms from each replica (Step A4) This will be biological replicate number 1 (replicates number 2 and 3 should be carried out in a different day).
3. Centrifuge worms for 2 min at 376 x g. Discard supernatant.
4. Fill with 1 ml of M9 + Lev.
5. Repeat Steps B3-B4, twice.
6. Discard M9+Lev and resuspend worms in 1 ml of M9 + Lev supplemented with Antibiotics (M9 + Lev + Ab).
7. Repeat Steps B3-B5, but with M9 + Lev + Ab, instead of M9 + Lev.
8. Discard supernatant, resuspend pelleted worms in 1 ml of M9 + Lev + Ab and incubate for one hour.
9. Repeat Steps B3-B5.

Note: Use just M9 + Lev.

10. Discard M9 + Lev as much as possible and lyse worms with a sterile pestle (could be motorized if available).

Note: Worms on each tube should be lysed with a sterile pestle. Lyse worms for 1 min, or until the worm pellet is completely dissolved.

11. Resuspend lysed worms in 500 µl of M9.

Note: Serial dilutions consist of series of successive measured dilutions that are prepared in order to reduce the concentration of bacteria and obtain a known number of colony forming units per sample.

12. Dilute 1:10 the worm lysate, in seven serial dilutions (dilution #1 to #7) in M9. For example, take 1 ml of worm lysate into 9 ml of M9 buffer. This will be dilution #1. Then, take 1 ml of the dilution #1 into 9 ml of M9 buffer, making dilution #2. Repeat sequentially until dilution #7.

13. Take 200 µl of dilution #5 (10^{-5}), #6 (10^{-6}), and #7 (10^{-7}), and plate them on solid LB with antibiotics. Streptomycin is used to select *E. coli* OP50, ampicillin is used to select fluorescent *Salmonella* Typhimurium MST1-GFP and *E. coli* OP50-GFP strains.

14. Incubate plates overnight at 37 °C.

C. Quantification of bacterial colonization by calculating Colony Forming Units (CFU) in *C. elegans* intestines (or simple plating)

Notes:

- a. Next day, check for undesired bacterial or fungi contamination ensuring you will count colonies

of bacteria with the reported morphology. For example, *E. coli* OP50 colonies are tiny, round, creamy white in color and with defined borders. In contrast, *Salmonella* colonies are shiny, with dense center and round margins.

- b. You will have 9 plates of isolated colonies from each bacterium, in this case, 18 plates in total (3 from *E. coli* replicates, and other three replicates from *Salmonella*, per each 10^{-5} , 10^{-6} , 10^{-7} dilution).
1. Count the number of colonies with a Hand Tally Counter (Humboldt). Digital counters, or image-based counters can also be used.
2. Register each value in an MS® Excel sheet.
3. Calculate the CFU per worm using the formula:

$$CFU \text{ per worm} = \frac{\left\{ Number \text{ of Colonies} * \left(\frac{1}{10^{\text{Dilution Factor}}} \right) * \text{Plated Volume (mL)} \right\}}{\text{Number of worms}}$$

- D. Quantification of the degree of bacterial colonization in the gut using fluorescence microscopy
 1. Prepare agar pads as described by Monica Driscoll (www.wormatlas.org/agarpad.htm) or in WormBook. Instead of a 5% agar solution, we use 2%.
 2. Pick 15 worms per bacterial condition to individual agar pads containing a drop of 1 mM levamisole hydrochloride using a platinum pick. Small amounts of playdough can be placed at each corner of a 22 x 22 coverslip before covering the preparation to avoid pressuring the worm.
 3. Classify worms according to the presence of GFP positive bacteria in their guts. As described in Palominos *et al.* (2017), worms with no detectable fluorescence or discrete bacteria in the pharynx are classified as “undetectable” (Figure 2A'). Worms with one-third of the intestine with fluorescent bacteria are classified as “partial”, and “full” when GFP tagged bacteria is found along the whole intestine (Figure 2B').
 4. Quantify the number of animals with different phenotypes. Save raw data.
 5. Take representative images of each phenotype.

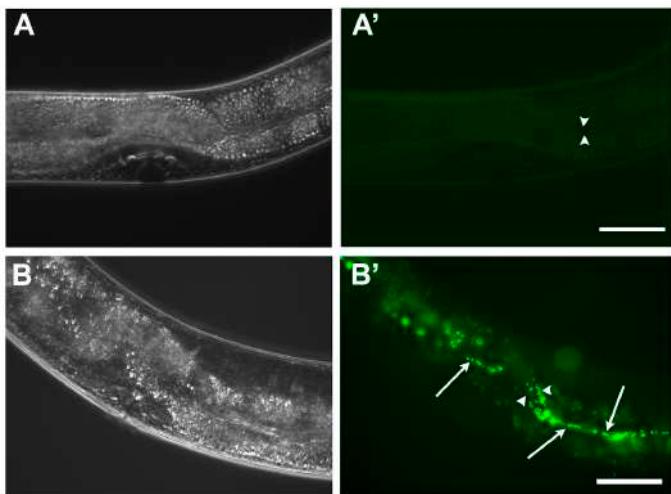


Figure 2. Intestinal colonization of *C. elegans* gut by fluorescent bacteria. A-A'. L4 wild type worm grown on *E. coli* OP50-GFP. Most worms on these bacteria possess just scattered bacteria in the pharyngeal grinder. B-B'. L4 wild type worm grown on *Salmonella* Typhimurium MST1-GFP. Worm fed on *Salmonella* presenting a “full” colonization phenotype, showing individual and clumped GFP positive bacteria (arrows) along the intestine. Moreover, intestinal expansion can be observed (arrowheads). Scale bars = 50 μ m.

Data analysis

1. Each experiment should be done at least three times (biological replicas, independent experiments done in different days) and in triplicates (three samples in each replica). See Palominos *et al.*, 2017, Materials and Methods for more information. Each biological replicate will be the numerical value corresponding to the average of the three triplicates.
2. One way of plotting the results, is by using Grouped Columns in Prism 6 software (GraphPad). Each column is the average of one biological replica.
3. For CFU calculations use Unpaired *t* test analysis, with 0.05 significance (Figure 3A).
4. For Intestinal colonization use two-way ANOVA, Holm-Sidak test, with 0.05 significance (Figure 3B).

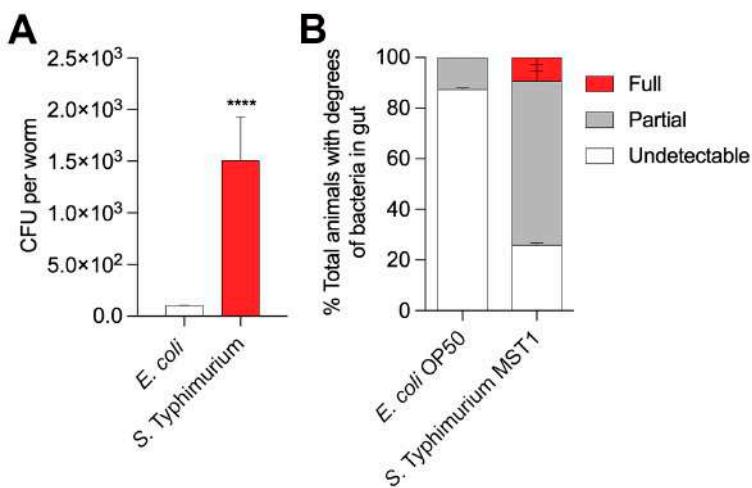


Figure 3. Two different assays to quantify intestinal colonization by bacteria in *Caenorhabditis elegans*. A. Colony Forming Units (CFU) per worm growing in *E. coli* OP50 (white) and *Salmonella* Typhimurium MST1 (red) for two generations. Unpaired *t*-test, $P < 0.0001$. N = 90 worms per biological replica, each column contains three replicates. B. Percentage of L4 worms showing different phenotypes of intestinal colonization when grown on *E. coli* OP50-GFP and *S. Typhimurium* MST1-GFP. Full, partial and undetectable are defined in Procedure D. Two-way ANOVA, Holm-Sidak test, $P < 0.05$. N = 45 worms per biological replica.

Notes

1. Worms selected for CFU analysis should be healthy. Avoid fungi or other kind of contamination before starting the experiments.
2. All solutions and containers should be sterile.
3. Intestinal colonization assays can be done using 60 mm plastic Petri dishes seeded with 150 μ l of bacterial culture (Procedure A).
4. Always select worms in the same developmental stage.
5. Autoclave plastic pestles if not sterile.

Recipes

1. Nematode Growth Medium (NGM) plates
 - a. Add the following to a 1 L Schott Bottle
 - 3 g NaCl
 - 2.5 g Bacto Peptona
 - 17 g Bacto Agar
 - Double distilled water (928 ml)
 - b. Stir bar
 - c. Autoclave for 20 min at 121 °C

- d. Place on stir plate, wait until cooled at around 55 °C
 - e. Add the following
 - 1 ml of 1 M CaCl₂ sterile
 - 1 ml of 1 M MgSO₄ sterile
 - 25 ml of 1 M KH₂PO₄ pH 6.0 sterile
 - 1 ml of 5 mg/ml cholesterol (prepared in 95% ethanol, and stored at RT)
 - f. Pour onto 90 mm sterile plates in a laminar flow cabinet
 - g. Let dry for one night
 - h. Seed with appropriate bacteria, or store at 4 °C
2. Solid Luria-Bertani (LB)
 - a. Dissolve 15 g of Bacto agar, 10 g of Bacto Tryptone, 10 g of NaCl and 5 g of Yeast extract in 1,000 ml dH₂O
 - b. Autoclave 20 min at 121 °C
 - c. Swirl it gently to distribute melted agar evenly through the solution. Be careful! Superheated liquids may boil over when swirled
 - d. Once cooled to 55 °C add antibiotics at 50 mg/ml (ampicillin and streptomycin)
 - e. Swirl avoiding bubbles
 - f. Set up a color/mark code (e.g., two red lines for LB-streptomycin, two black lines for LB-ampicillin plates)
 - g. Pour around 30-35 ml per plate
 - h. When medium has hardened completely, invert and store them at 4 °C until needed
 - i. Remove plates for storage 1-2 h before using them
 3. Liquid LB
 - a. Dissolve 10 g of Bacto Tryptone, 10 g of NaCl and 5 g of Yeast extract in 1,000 ml
 - b. Autoclave 20 min at 121 °C
 - c. Once cooled to 55 °C add antibiotics at 50 mg/ml (ampicillin and streptomycin)
 4. Sterile 1 M MgSO₄ solution
 - a. Dissolve 123.24 g MgSO₄·7H₂O in 500 ml of pure MilliQ water
 - b. Autoclave at 121 °C for 20 min
 - c. Store at room temperature (RT)
 5. Sterile 1 M CaCl₂
 - a. Dissolve 5.55 g of CaCl₂ dehydrate in 50 ml of MilliQ water
 - b. Autoclave
 - c. Store at RT
 6. Sterile Phosphate buffer
 - a. Dissolve 10.7 g of K₂HPO₄ and 32.5 g of KH₂PO₄ to 300 ml of MilliQ water
 - b. Adjust pH to 6.0
 - c. Autoclave
 - d. Store at RT

7. M9 buffer

- a. Dissolve the following in 1 L of pure MilliQ water
 - 3 g KH₂PO₄
 - 6 g Na₂HPO₄
 - 5 g NaCl
- b. Autoclave at 121 °C for 20 min, then add 1 ml of sterilized 1 M MgSO₄ solution
- c. Store at RT

Note: Open just next to the flame, on a sterile hood, with gloves, as quickly as possible.

8. Levamisole 250 mM (stock) in M9

- a. Mix 9.03 g of levamisole hydrochloride in 15 ml of sterile M9
- b. Stir if necessary
- c. Aliquot in 1 ml microcentrifuge tubes
- d. Keep it at -20 °C. Avoid re-thawing

9. M9 + Lev (M9 with 25 mM levamisole)

- a. Mix 5 ml of 250 mM levamisole stock solution with 45 ml of sterile M9
- b. Keep it on ice

10. M9 + Lev + Ab (M9 with 25 mM levamisole with antibiotics)

- a. Prepare 5 ml of Gentamicin-Ampicillin stock solution (10 mg/ml) by dissolving 50 mg of gentamicin and 50 mg ampicillin in 5 ml of MilliQ water.
- b. Filter with a 0.2 µm syringe filter. Keep it on ice until finishing aliquoting. Store at -20 °C
- c. Use 2,000 µl of the stock Gen-Amp (10 mg/ml) and mix with 18 ml of M9 + Lev. Keep it on ice until used

11. 1 mM levamisole for microscopy

- a. Mix 40 µl of M9 + Lev with 960 µl of M9
- b. Filter with a 0.2 µm syringe filter
- c. Store at 4 °C. Keep on ice when using

12. 87% glycerol for bacterial stocks

- a. Mix 8.7 ml of 100% glycerol in 1.3 ml of MilliQ water
- b. Autoclave
- c. Store at room temperature, covered from light

13. 2% agar for microscopy

- a. Prepare 3 ml of 5% agar/M9 in a 5 ml glass culture tube
- b. Place the glass culture tube with 2% agarose inside a 500 ml glass beaker, with 150-200 ml of RT water (as imitating a water bath)
- c. Microwave for 60 s as medium potency

Note: Check homogeneity of the mixture. Resuspend with a plastic pipette if necessary. Be sure that the agarose is completely melted before going to the next step.

- d. Before making the pads keep 2% agar at 60 °C in a heating block or bath
- e. After use, store it at 4 °C

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Competing interests

The authors declare they have no financial interest.

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HIV-CRISPR: A CRISPR/Cas9 Screening Method to Identify Genes Affecting HIV Replication

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[Abstract] Screening with CRISPR/Cas9 technology has already led to significant discoveries in the fields of cancer biology, cell biology and virology. Because of the relatively low false discovery rates and the ability to perform high-throughput, pooled approaches, it has rapidly become the assay of choice for screening studies, including whole-genome screens. Here, we describe a CRISPR screening protocol that allows for efficient screening of the entire life cycle of HIV-1 through packaging of the HIV-CRISPR lentiviral genomes by infecting HIV-1 virus *in trans*.

Keywords: HIV, CRISPR screen, Cofactors, Restriction factor, HIV-CRISPR, High-Throughput Sequencing (HTS)

[Background] Genetic screens are powerful tools to identify new genes affecting the replication of viruses, including Human Immunodeficiency Virus (HIV). Identification of host genes important in HIV infection allows for enhanced understanding of HIV replication, evolution, transmission and pathogenesis, key interests of the HIV field. In particular, the discovery of HIV restriction factors, which are often Interferon (IFN) Stimulated Genes (ISGs), has been a crucial development in the field of retrovirology in the last decade. High-throughput approaches to discover HIV restriction factors have focused on over-expression screens to identify broadly acting antiviral ISGs (Schoggins *et al.*, 2011) or acting specifically against HIV (Kane *et al.*, 2016). One whole-genome screen for HIV restriction factors was performed by transfecting pools of siRNA pools in target cells (Liu *et al.*, 2011). However, these methods lacked the robustness, versatility, and high-throughput aspects of CRISPR/Cas9 genetic screens. Thus, we developed an innovative approach using the CRISPR/Cas9 technology and relying on the ability of HIV to cross-package lentiviral genomes expressed in cells transduced with CRISPR/Cas9 libraries (OhAinle *et al.*, 2018). This protocol was used originally to discover ISGs that block HIV-1 infection in the monocytic cell line THP-1, using a library of 2000 ISGs we designed. However, it can be used with any single guide RNA (sgRNA) library (whole-genome or subsets of genes) that is synthesized and assembled into the HIV-CRISPR lentiviral backbone. HIV-CRISPR was constructed by replacing the U3-deleted Self-Inactivating (SIN) LTR in the lentiCRISPRv2 vector with the full-length LTR from HIV-1_{LAI}. This modification allows for HIV-CRISPR genomic RNA to be transcribed and available for packaging *in trans* into HIV virions. It can be used with any human cell line permissive for lentiviral transduction and editing, and can be used to study a variety of HIV isolates, mutants or even other closely-related lentiviruses that can package the HIV-CRISPR lentiviral genomes *in trans*. Importantly, this protocol assesses key factors important to the life cycle of HIV, including late

steps such as assembly or budding, which may not be examined by other approaches relying on the use of reporter viruses.

Materials and Reagents

1. 6-well and 12-well tissue culture plates
2. 10 cm tissue culture dishes
3. T-75 tissue culture flasks
4. 0.22 µm filter flasks [such as MilliporeSigma™ Stericup™ Quick Release-HV Vacuum Filtration System (0.45 pore)] (Fisher, catalog number: SCHVU02RE)
5. 0.22 µm filters (such as MilliporeSigma™ Millex™-GP Sterile Syringe Filters with PES Membrane) (Fisher, catalog number: SLGP033RS)
6. Steriflip 50 ml conical tubes (MilliporeSigma™ Steriflip™ Sterile Disposable Vacuum Filter Units) (Fisher, catalog number: SCGP00525)
7. 50 ml conical tubes
8. Filter/barrier and non-filter/barrier tips
9. Serological pipettes
10. 293T cells (ATCC, catalog number: CRL-3216), grow in complete DMEM: DMEM 10% FBS, 1% Pen/Strep
11. THP-1 cells (NIH AIDS reagent program, catalog number: 9942; RRID, catalog number: CVCL_0006), grow in complete RPMI: RPMI 10% FBS, 1% Pen/Strep, 10 mM HEPES, Glutamax
12. TZM-bl cells (NIH AIDS reagent program, catalog number: 8129), grow in complete DMEM: DMEM 10%FBS, 1% Pen/Strep
13. HIV-CRISPR plasmid (NIH AIDS reagent program, catalog number: 13567)
14. HIV-CRISPR Library (PIKA-HIV library, NIH AIDS reagent program, catalog number: 13566)
15. HIV vector packaging and envelope plasmids: pMD2.G plasmid (Addgene, catalog number: 12259) and psPAX2 plasmid (Addgene, catalog number: 12260)
16. Fetal Bovine Serum, heat-inactivated (Peak Serum, catalog number: PS-FB2 or equivalent)
17. DMEM (Gibco, catalog number: 11965-092)
18. RPMI (Gibco, catalog number: 11875-093)
19. Penicillin/streptomycin (Gibco, catalog number: 15140-122 or equivalent)
20. HEPES (Gibco, catalog number: 15630-080)
21. Glutamax (Gibco, catalog number: 35050-061)
22. Mirus Bio *TransIT-LT1* Transfection Reagent (Fisher Scientific, catalog number: MIR2305)
23. Bovine Serum Albumin (Sigma, catalog number: A1933)
24. Puromycin dihydrochloride (Sigma, catalog number: P8833)
25. HIV viral stock, such as HIV_{LAI} (BRU) Viral Stock (NIH AIDS reagent Program, catalog number: 2522)

26. DEAE-Dextran hydrochloride (Sigma, catalog number: D9885)
27. Qubit dsDNA HS Assay Kit (Thermo Fisher, catalog number: Q32854)
28. QIAamp Viral RNA Mini Kit (Qiagen, catalog number: 52904)
29. QIAamp DNA Blood Midi Kit (Qiagen, catalog number: 51185)
30. QIAquick PCR Purification Kit (Qiagen, catalog number: 28104)
31. RNase AWAY Decontamination Reagent (Thermo Fisher, catalog number: 10328011)
32. SuperScript II Reverse Transcriptase (Thermo Fisher, catalog number: 18064014)
33. Herculase II Fusion DNA Polymerase (Agilent, catalog number: 600679)
34. PCR oligos (see [Supplemental](#))

Oligos for the PCR1 reaction are custom DNA oligos from IDT, with standard purification/desalting (or equivalent). The PCR2 reaction oligos (R2_R, R2_Indexes) are HPLC-purified ultramers from IDT (or equivalent).

35. dNTPs (Thermo Fisher, catalog number: R0192)
36. DMSO (Sigma, catalog number: 472301)
37. Nuclease-free H₂O
38. Agarose
39. Agencourt AMPure XP Beads (Beckman Coulter Life Sciences, catalog number: A63880)
40. HiSeq Rapid SBS Kit v2 (Illumina, catalog number: FC-402-4022)
41. HiSeq Rapid Cluster Kit v2 (Illumina, catalog number: GF-402-4002)
42. MiSeq Reagent Kit v2 (Illumina, catalog number: MS-102-2001)
43. PhiX Control V3 Kit (Illumina, catalog number: FC-110-3001)
44. Tris base (Sigma, catalog number: 10708976001)
45. Boric acid (Sigma, catalog number: B6768)
46. 0.5 M EDTA (Sigma, catalog number: E9884)
47. 10x TBE running buffer (see Recipes)

Optional:

1. Human IFN Alpha Hybrid (Universal Type I Interferon) (PBL assay science, catalog number: 11200-1). Dilute in PBS 0.1% BSA and keep aliquots at -80 °C
2. Sucrose
3. NaCl
4. 20% sucrose solution (see Recipes)

Equipment

1. SW28 Beckman Polyallomer ultracentrifuge tubes (Beckman Coulter, catalog number: 326823)
2. Microcentrifuge (Eppendorf, model: 5425R or equivalent)
3. Ultracentrifuge (Beckman Coulter, model: Optima L-90K or equivalent)
4. SW28 (Eppendorf, catalog number: 342207) or SW32Ti (Beckman Coulter, catalog number:

- 369694) Ultracentrifugation rotor (or equivalent)
5. Eppendorf 5810 tabletop centrifuge (Eppendorf, catalog number: 022625004)
 6. Swinging-bucket rotor (Rotor S-4-104) (Eppendorf, catalog number: 5820755008)
 7. Swinging-bucket rotor plate adapters (Eppendorf, catalog number: 022638866)
 8. Vortex
 9. Water baths
 10. Nanodrop
 11. Applied Biosystems ProFlex Thermal Cycler (or equivalent)
 12. Magnetic rack (96-well)
 13. Qubit 4 Fluorometer (Thermo Fisher)
 14. Illumina MiSeq, HiSeq or equivalent
 15. CO₂ tissue culture incubator
 16. -80 °C freezer

Software

1. MAGeCK: <https://sourceforge.net/p/mageck/wiki/Home/>
2. Bowtie: <http://bowtie-bio.sourceforge.net/index.shtml>
3. FASTX Toolkit Barcode Splitter: http://hannonlab.cshl.edu/fastx_toolkit/

Procedure

Overview of the procedure (Figure 1):

- Preparation of HIV-CRISPR Library Lentiviral Vector Stock (A)
- Quantification of the HIV-CRISPR Lentiviral Vector Stock Titer (B)
- HIV-CRISPR Library Transduction (C)
- HIV-CRISPR Library Cell Infection (D)
- HIV-CRISPR Cellular Genomic DNA Extraction (E)
- HIV-CRISPR Viral RNA Extraction (F)
- Cellular Genomic DNA HIV-CRISPR Library Amplification (G)
- Viral RNA HIV-CRISPR Library Amplification (H)
- Library Clean-Up: Double-Sided SPRI (I)
- Quantification of Libraries (J)
- Pooling libraries for High-Throughput Sequencing (K)
- High-Throughput Sequencing (HTS) of HIV-CRISPR Libraries (L)
- HIV-CRISPR Screen Analysis (M)

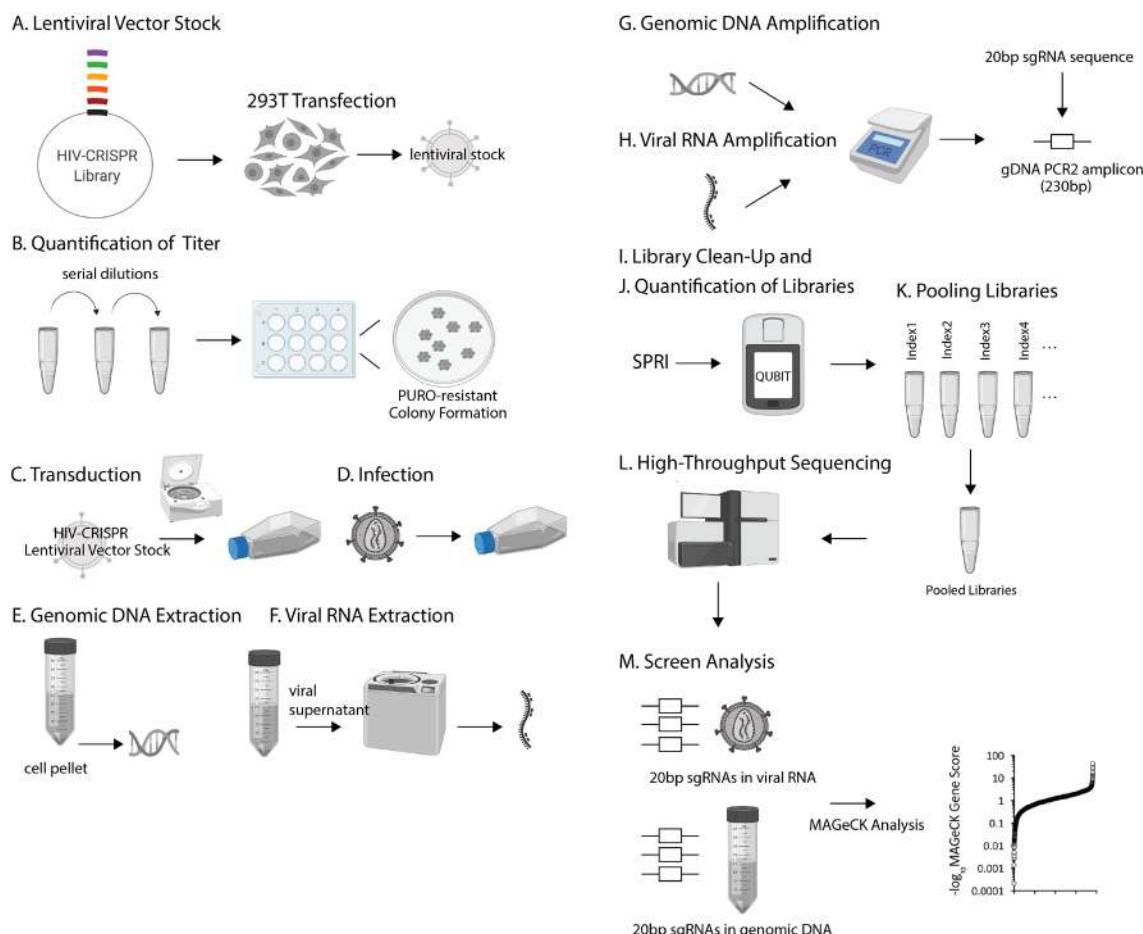


Figure 1. Schematic of the overall HIV-CRISPR Screening procedure

A. Preparation of HIV-CRISPR Library Lentiviral Vector Stock

The goal of Procedure A is to generate a library-scale HIV-CRISPR lentiviral vector stock to be used to create HIV-CRISPR cells (Procedure C).

1. Day 0: plating 293T cells

One day prior to transfection seed 293T cells at a concentration of 2×10^5 cells/ml in 2 ml/well in 6-well plates. Use low passage 293T cells maintained below confluence for several passages in order to increase lentiviral titers. Typically, 20 6-well plates are prepared at a time and are usually enough for several transductions of a ~15,000 sgRNA library or one transduction with a ~100,000 sgRNA whole-genome library.

2. Day 1: transfection

- Check the confluence of cells: ideally, they should be at 50% confluence at the time of transfection.
- For each well of a 6-well plate, mix 667 ng of the HIV-CRISPR Library plasmid, 500 ng of the psPAX2 plasmid (HIV GagPol) and 333 ng of the pMD2.G plasmid (VSV-G).
- Add all DNA to 200 μ l of serum-free medium (SFM-DMEM) and vortex for 5 s.
- Bring the Transit-LT1 transfection reagent to room temperature and vortex for 5 s, making sure that no precipitate is visible.

- e. Add 4.5 μ l TransIT-LT1 (ratio of 3 μ l MIRUS to 1 μ g of total plasmid DNA) directly to SFM media/DNA and vortex for 10 s.
 - f. Incubate the DNA/TransIT-LT1 mix for 20 min to form complexes.
 - g. Add transfection mix slowly to each well of 293T cells and gently swirl the plate to distribute.
3. Day 2: replace transfection media
Remove the media and replace it with 1.5 ml of complete DMEM per well.
4. Day 3: collect viral supernatant
 - a. Remove viral supernatant from cells.
Optional: replace media with 1.5 ml of complete DMEM per well for a second collection the following day.
 - b. Clarify supernatant by centrifuging cell debris for 5 min at 1,000 $\times g$ at room temperature.

Note: All centrifugation steps are carried out at room temperature (23 °C) unless otherwise specified.

 - c. Collect cleared viral supernatant in a new 50 ml Falcon tube.
 - d. (Optional) Day 4: Collect the viral supernatant, similarly as on Day 3. Pool the supernatants from collection 1 and 2 together.

5. Filtering: Filter viral supernatant with a 0.22 μ m filter flask.

Note: Supernatants can be stored at 4 °C overnight or for several days without significant loss of infectivity.

 6. Virus concentration: Add 33 ml of viral supernatant to SW28 Beckman Polyallomer ultracentrifuge tubes. If less than 33 ml viral supernatant is available, fill with DMEM media. Carefully add 4 ml of 20% sucrose solution to each SW28 Beckman Polyallomer ultracentrifuge tube with a 5 ml pipet. The tube should be filled up to 0.5 cm from the top to avoid collapsing during ultracentrifugation. Finish filling the tube with complete DMEM media as needed.
 7. Load the SW28 ultracentrifuge tubes into a pre-chilled SW28 or SW32Ti rotor. Spin at 70,000 $\times g$ for 1 h at 4 °C.
 8. Decant clarified viral supernatants and let tubes dry inverted on a paper towel for several minutes. Put each tube into a 50 ml conical tube and resuspend the (non-visible) pellets in the desired volume (from 200 μ l to 1 ml) and incubate at 4 °C.
 9. After 15-60 min of incubation, vortex gently for 5 s. Incubate at 4 °C for several hours (or ideally overnight) with occasional vortexing.
 10. Gently resuspend viral pellets in DMEM media using a 1 ml pipet (avoid generating bubbles), combine all supernatants, mix, aliquot and freeze at -80 °C. Small aliquots (20-50 μ l) can be made for titering in TZM-bl or other cells.

Notes:

- a. Scale up the transfection as needed, depending on the amount of lentivector needed.
- b. Lentiviral vector production is more efficient in 6-well plates rather than 10 cm or other dishes.
- c. All waste should be handled according to institutional biosafety rules.

B. Quantification of the HIV-CRISPR Lentiviral Vector Stock Titer

The aim of this step is to quantify the titer of the lentiviral vector stock produced in Procedure A. The Colony Forming Assay used here will allow calculating the volume of lentiviral vector stock necessary to achieve the desired Multiplicity of Infection (MOI). Multiplicity of Infection (MOI) is a ratio of infectious particles to the number of cells and can be used to deliver a specific number of transducing lentiviral vectors on average to cells in culture.

1. Day 0: cell plating

Plate TZM-bl cells at 1.5×10^5 cells/ml in a 12-well plate (1 ml per well).

2. Day 1: virus infections

- a. Replace the media with 300 μ l of complete DMEM, with a final concentration of 20 μ g/ml DEAE-Dextran.
- b. Thaw an aliquot of the HIV-CRISPR lentivector preparation.
- c. Prepare the following HIV-CRISPR lentivector dilutions in duplicate in complete DMEM: 1:10, 1:100, 1:1,000, 1:3,000, 1:10,000 (for example, dilute 20 μ l to 180 μ l for a 1:10 dilution, serial dilute from there)
- d. Add 20 μ l of diluted lentivector per well of a 12-well plate in duplicate.
- e. Infect cells by spin-infection (spinoculation) for 30 min at $1,100 \times g$ at room temperature, using a tabletop centrifuge with swinging-bucket rotors/plate adapters.
- f. Add 200 μ l media to well to fill to 500 μ l per well. Incubate cells overnight at 37 °C in a CO₂ incubator.

3. Day 2: puromycin selection

Replace media with complete DMEM with 0.5 μ g/ml puromycin (1 ml per well).

Note: Puromycin concentration is cell-type dependent and can be determined with a drug kill curve (see Step C2c).

4. Day 3-Day 11: cell killing

- a. Replace the media with fresh Puromycin-containing DMEM media as needed to clear off dead cells until colonies of cells are visible and can be manually counted (typically in the 1:1,000-1:3,000 range).
- b. Calculate the titer (Infectious Units/ml of lentiviral supernatant). For example, if 40 colonies are counted (on average) at the 1:1,000 dilution, this would be equivalent to 40,000 IU for 20 μ l of inoculum used in the titering assay. This is equal to 2×10^6 IU/ml in the lentiviral supernatant.

C. HIV-CRISPR Library Transduction

This step consists in the generation of a pool of cells stably expressing the Cas9 enzyme and the sgRNA library. Upon transduction with the lentiviral stock produced in Procedure A and quantified in Procedure B, each target cell will carry an integrated lentiviral HIV-CRISPR lentiviral genome encoding for Puromycin resistance, the Cas9 enzyme, and a specific sgRNA from the library.

1. Day 0: cell plating and transduction

- a. Plate the cells to be transduced in a 12-well plate. For adherent cells, cells should be at approximately 75% confluence. For suspension cells, use a concentration of 2×10^6 cells per well in 500 μl in a 12-well.

Note: Plate enough cells to ensure 500x coverage of the library, meaning that each sgRNA in the library is sampled a minimum of 500 times. To calculate the number of cells to transduce, multiply the number of individual sgRNAs in the library by 500. For example, the GeCKOv2 whole genome library that contains 123,411 sgRNAs: therefore, 62×10^6 transduced cells should be targeted.

- b. Add DEAE-Dextran to the media to make a final concentration of 20 $\mu\text{g/ml}$ to enhance transduction efficiency.

Note: This concentration of DEAE-Dextran is optimal to enhance transduction of THP-1 cells. For other cell types, optimize this concentration with a dose response to ensure optimal transduction rates while minimizing cell toxicity.

- c. Add the appropriate volume of HIV-CRISPR library lentiviral vector to reach an MOI of 0.5-0.75, thereby allowing for a cell population post-selection in which only one sgRNA (and therefore gene knockout) is present in most cells in the population.
- d. Infect cells by spin-infection (spinoculation) for 30 min at $1,100 \times g$ at room temperature in a tabletop centrifuge with swing bucket rotors/plate adapters. Add 500 μl of media to each well and incubate overnight at 37 °C in a CO₂ incubator.

Notes:

- i. The amount of lentiviral stock to use in order to achieve an effective MOI of 0.5-0.75 may differ significantly in other cell types, as the rate of lentiviral transduction efficiency varies. Optimization of this step can be performed for each cell line used. Testing different amounts of DEAE-dextran and various dilutions of the lentiviral stock allows for cell type-specific determination of optimal transduction conditions.
- ii. Leftover lentivector stock not used at this step may be frozen back once without major loss of titer.

2. Day 1

- a. For suspension cells, combine all wells of the transduced cells in one tube by resuspending the cells with a 1 ml pipet (pipet up and down to fully resuspend all cells from each well) and collecting in a Falcon tube. Make sure that no cells stay attached to the bottom of the well. For adherent cells, trypsinize the cells to remove the cells from the plate.
- b. Pellet cells for 5 min at $1,000 \times g$.

- c. Resuspend cell pellet at a final concentration of 5×10^5 cells/ml in complete RPMI (or other media as appropriate) with 0.5 $\mu\text{g/ml}$ Puromycin (or other appropriate concentration).

Note: This concentration of Puromycin was optimized for selection in THP-1 cells, and other concentration as appropriate for each cell type. We recommend using puromycin concentrations previously published in the literature for each cell type of interest, or as determined by a kill curve prior to the transduction. The kill curve is performed by adding

increasing concentrations of puromycin on non-transduced cells, which allows determination of the minimal concentration necessary to kill 100% of cells after several days of puromycin selection in culture.

3. Day 2-Day 14: HIV-CRISPR cell library selection
 - a. Continue puromycin selection for 10-14 days to select for transduced cells and to allow for CRISPR/Cas9-mediated gene knockout to occur.
 - b. Replace the media every 2-3 days with complete media containing puromycin to expand the cells, always maintaining the cells under selection. Split cells when appropriate, always maintaining enough cells to ensure good coverage of the library (> 500x).
 - c. When selection is complete, use cells for the experiment and freeze leftover cells in 90% FBS and 10% DMSO for future experiments.

D. HIV-CRISPR Library Cell Infection

Cells generated in Procedure C are infected by HIV in order to assess the effect of each single gene knock-out in the library of cells on HIV replication. Typically, a screen for each condition (*i.e.*, virus) is done in duplicate to enhance screen results.

1. Day 0

Count and plate cells at 2×10^6 cells/ml in 500 μ l in 12-well dishes.

Note: Cells can be pretreated one day prior with 1000 U/ml of recombinant Universal Type I IFN alpha for 24 h.

2. Day 1

- a. Infect cells in a 12-well plate with 20 μ g/ml DEAE-Dextran. Different MOIs may be used depending on the aim of the experiment (identification of cofactors or restriction factors; sensitivity of the phenotype to saturation, low infection levels, etc.). Total volume for infection can vary, smaller overall volumes give higher rates of infection.
- b. Infect cells by spin-infection (spinoculation) for 30 min at 1,100 $\times g$ at room temperature, using a tabletop centrifuge with swinging-bucket rotors/plate adapters.
- c. Add 500 μ l of media in each well and incubate overnight at 37 °C in a CO₂ incubator.

3. Day 2

Spin cells for 5 min at 1,000 $\times g$ and resuspend them in media (with or without IFN as appropriate).

4. Day 4

Collect cells and supernatants for nucleic extraction, amplification and sequencing.

Notes:

- a. *Depending on the cell line or the desired outcome, different MOIs or time of collection can be used.*
- b. *Important for remaining protocol steps: contamination with plasmid DNA, genomic DNA or amplified libraries can be a problem and steps should be taken to reduce the likelihood of*

contamination. Use clean pipets with filter tips, freshly opened kits and reagents, and dedicated hoods and benches for each step as possible.

E. HIV-CRISPR Cellular Genomic DNA Extraction

Cellular genomic DNA is extracted from cells to allow subsequent PCR amplification (Procedure G) of the HIV-CRISPR lentiviral genomes (including the sgRNAs) integrated in the cell population.

1. Pellet cells by spinning for 5 min at 1,000 $\times g$ to separate cells and virus-containing supernatant. Save supernatants for viral RNA extraction (Procedure F). The pellets can be stored at 4 °C for a few days (or frozen at -80 °C although lysis becomes more difficult) before DNA extraction.
2. Extract genomic DNA (gDNA) using the QIAamp DNA Blood Midi Kit as per the protocol, with the following modifications:
 - a. Ideally use a maximum of 5×10^6 cells per extraction (scale up to multiple extractions as required).
 - b. Perform the extra centrifugation step to remove as much ethanol as possible before eluting (ethanol can inhibit downstream amplification steps).
 - c. Elute genomic DNA in 250 μl H₂O instead of the buffer provided in the DNA Blood Midi kit (which contains EDTA and can inhibit downstream amplification steps).
 - d. Perform two successive 250 μl elutions. Keep Elution 1 and Elution 2 separate, and quantify DNA using a nanodrop (typical yield is 100-300 ng/ μl). The second elution is usually less concentrated, but more efficiently amplified because of reduced ethanol carry-over.
 - e. Freeze gDNA at -20 °C or -80 °C.

F. HIV-CRISPR Viral RNA Extraction

HIV virions are collected from the supernatant and purified. Viral RNA from virions is extracted to allow subsequent PCR amplification of the sgRNA encoded by the HIV-CRISPR viral genomic RNA.

1. Collect viral supernatant when separating cells in Procedure E above. Viral supernatant can be stored for up to a week at 4 °C before or after filtering (see Procedure A or long-term at -80 °C).
2. Filter supernatants with a 0.22 μm filter or with a Steriflip 50 ml conical tube.
3. Concentrate viral particles over a 20% sucrose solution, as described in Step A6. The centrifugation is carried out at 70,000 $\times g$ for 1 h at 4 °C.
4. Slowly resuspend the viral pellets in 140 μl of PBS, and incubate overnight at 4 °C, occasionally vortexing.

Note: This step can be performed after several hours at 4 °C.

5. Transfer to a 1.5 ml Eppendorf tube. Store at 4 °C for several days or freeze at -80 °C if not used right away for RNA extraction.
6. Extract viral RNA using the QIAamp Viral RNA Mini Kit as per the protocol, with the following modifications:
 - a. This step should be carried on a dedicated RNA bench. Clean the bench and pipettes thoroughly with RNase away. Use filter/barrier tips.

- b. The carrier RNA is not added to Buffer AVL during lysis.
 - c. Viral RNA (vRNA) is eluted in 40 µl of buffer AVE to facilitate downstream reverse transcription (RT) reactions.
7. Freeze vRNA at -80 °C and avoid multiple freeze/thaw cycles.

G. Cellular Genomic DNA HIV-CRISPR Library Amplification

sgRNA sequences present in the integrated HIV-CRISPR vectors in the pool of cells are amplified by PCR from cellular genomic DNA (Procedure E). Primers for amplification flank the variable sgRNA sequences, resulting in unbiased amplification of all sgRNA sequences present in the HIV-CRISPR Cell Library. The library representation of sgRNA sequences in the cellular genomic DNA is used to normalize the data during Screen Data Analysis (Procedure L).

1. Thaw isolated genomic DNA (gDNA) preps (from Procedure E) and PCR reagents on ice.
2. Calculate the amount of gDNA to amplify to ensure approximately 500x coverage of the library (using an estimate of 6.6 pg of total genomic DNA per cell on average) and the number of PCR reactions to perform, using a maximum of 2 µg of genomic DNA for each PCR reaction to ensure most efficient amplification. For example, for the GeCKO v2 library containing 123,411 sgRNAs, a 500x coverage corresponds to 6.17×10^7 cells, or 410 µg of DNA. In this case, perform 205 PCR reactions.
3. Perform PCR on the HIV-CRISPR lentiviral genome, to obtain a PCR1 product of 445 bp (Figure 2). PCR reactions are set up as follows (Table 1):

Notes:

- a. *The list of primers used is available in [Supplemental](#).*
- b. *Preparation of PCR reactions is done in a dedicated, DNA-free clean area or hood with dedicated, plasmid-free pipettes to avoid contamination during amplification.*

Table 1. First-Round PCR (PCR1)

	Component	50 µl reaction
PCR Reaction	gDNA (from Procedure E)	2 µg
Setup	R1_forward primer (10 µM)	1.25 µl
	R1_reverse primer (10 µM)	1.25 µl
	100 mM dNTPs	0.5 µl
	Buffer	10 µl
	DMSO	2.5 µl
	Herculase	1 µl
	H ₂ O	up to 50 µl
Thermocycling condition	95 °C	2 min
	95 °C	15 s
	60 °C	20 s
	72 °C	30 s
	72 °C	3 min
	4 °C	hold

4. Combine together all 50 µl PCR1 reactions in a single tube.

Note: The total number of PCR1 reactions to be pooled at this step will vary, depending on the total amount of genomic DNA amplified (as determined in Step G2).

5. Clean up the PCR1 pool with the QIAquick PCR clean up kit, following the kit instructions:

http://2012.igem.org/wiki/images/a/a3/QIAquick_PCR-purification.pdf.

Note: We typically pool up to 12 PCR reactions on a single column and elute in 50 µl of H₂O. This allows for concentration of the PCR1 product, while reducing the number of individual columns needed and avoiding saturation of the columns (that can bind up to 10 µg of DNA according to the manufacturer's instructions).

6. Combine the cleaned up PCR1 reactions to create a cleaned-up PCR1 Pool.

7. Perform the PCR2 reaction, leading to amplification of a PCR2 product (size = 230 bp). PCR reactions are set up as follows (Table 2):

Notes:

- Four PCR2 reactions are performed to amplify sufficient product for clean -up and pooling for HTS while also avoiding PCR bias that could be introduced in a single PCR reaction.*
- Each sample (genomic DNA replicate) must be amplified with a unique barcoded Indexing Primer (these are the "R2_IndexX" primer set). Extra care should be taken to avoid cross-contamination of Indexing Primers as this would result in mis-assignment of reads during barcode demultiplexing. The list of primers used is available in [Supplemental](#). Other custom primer sets can be designed and used if desired.*

Table 2. Second-Round PCR (PCR2)

	Component	50 µl reaction
PCR Reaction	gDNA PCR1 Pool	5 µl
Setup	R2_indexX primer (10 µM)	1.25 µl
	R2_reverse primer (10 µM)	1.25 µl
	100 mM dNTPs	0.5 µl
	Buffer	10 µl
	DMSO	2.5 µl
	Herculase	0.5 µl
	H ₂ O	29 µl
Thermocycling condition	95 °C	2 min
	95 °C	15 s
	60 °C	20 s
	72 °C	30 s
	72 °C	3 min
	4 °C	hold

8. PCR amplification can be verified by running a small amount of the PCR2 reactions on a 2% analytical TBE gel (230 bp product).

Notes:

- Carried-through PCR1 product (the 445 bp product amplified in the first round of PCR) may remain visible but should not affect downstream pooling and HTS as most of this product is removed during the Library Clean Up (Procedure I). Carried through product could affect accurate quantification but does not contain sequences necessary for HTS and, therefore, do not interfere with sequencing (Procedure K).*
- TBE (as opposed to TAE) allows for easier visualization of the 230 bp amplicons but should not be used for gel purification.*



Figure 2. Round 1 (PCR1) and Round 2 (PCR2) amplicons. A. A zoomed view of the HIV-CRISPR sequence is shown with the R1 (PCR1) and R2 (PCR2) primer binding sites mapped. An example 20 bp sgRNA sequence is displayed in the HIV-CRISPR vector backbone. B. Example gel showing expected PCR2 amplicon (230 bp). Note that some 445 bp PCR1 amplicon product is also visible.

9. Combine together all PCR2 reactions to create the gDNA PCR2 Pool. This includes all four PCR2 reactions, as indicated in Step G7.
10. Freeze the gDNA PCR2 Pool at -20 °C or at 80 °C.

H. Viral RNA HIV-CRISPR Library Amplification

The sgRNAs encoded in the packaged HIV-CRISPR viral genomes (packaged *in trans* by HIV) are amplified by PCR. This allows for the relative frequency of each sgRNA in the virions to be measured.

Note: Preparation of PCR reactions are done in a dedicated, DNA-free clean area or hood with dedicated, plasmid-free pipettes to avoid contamination during amplification.

1. Thaw viral RNA (vRNA from Procedure F), the SuperScript II Reverse Transcriptase kit and Herculase PCR reagents on ice.
2. Perform reverse transcription of viral RNA as follows (Table 3):
Note: The list of primers used is available in [Supplemental](#).

Table 3. RT

Component	20 µl reaction
vRNA (from Procedure F)	10 µl
R1_reverse primer (10 µM)	1 µl
10 mM dNTPs	1 µl
5x FS Buffer	4 µl
0.1M DTT	2 µl
RNaseOUT	1 µl

- Incubate RT reactions for 5 min at 65 °C in a Thermal Cycler, then place on ice.
- Add 1 µl of the Superscript II enzyme to each RT reaction.

Note: Mix well by pipetting up and down carefully to avoid bubbles. This step is key to ensure adequate mixing of reagents for maximum-efficiency amplification of all HIV-CRISPR viral RNA templates.

- Incubate in a Thermal Cycler as follows:
42 °C 50 min
70 °C 15 min
- Pool together all RT reactions to create the vRNA RT Pool.
- Next, PCR1 amplification of the reverse-transcribed viral RNA cDNAs is performed similar to genomic DNA amplification (Table 4).

Table 4. First-Round PCR for RT (PCR1)

	Component	50 µl reaction
PCR Reaction	vRNA RT Pool	10 µl
Setup	R1_forward primer (10 µM)	1.25 µl
	R1_reverse primer (10 µM)	1.25 µl
	100 mM dNTPs	0.5 µl
	Buffer	10 µl
	DMSO	2.5 µl
	Herculase	1 µl
	H ₂ O	23.5 µl
Thermocycling condition	95 °C	2 min
	95 °C	15 s
	60 °C	20 s
	72 °C	30 s
	72 °C	3 min
	4 °C	hold

- Up to 8 PCR1 reactions are pooled together and cleaned up using a single column of the QIAquick PCR clean up kit, with a 50 µl final elution volume (vRNA PCR1 Pool).

9. 12.5 μ l of the vRNA PCR1 Pool is used as template in four PCR2 reactions (Table 5):

Notes:

- a. *Each sample (vRNA replicate) must be amplified with a unique barcoded Indexing Primer (these are the “R2_IndexX” primer set). Extra care should be taken to avoid cross-contamination of Indexing Primers as this would result in mis-assignment of reads during barcode demultiplexing. The list of primers used is available in [Supplemental](#). Other custom primer sets can be designed and used if desired.*
- b. *Four PCR2 reactions are performed to amplify sufficient product for clean -up and pooling for HTS while also avoiding PCR bias that could be introduced in a single PCR reaction.*

Table 5. Second-Round PCR (PCR2)

	Component	50 μ l reaction
PCR Reaction	vRNA PCR1 pool	12.5 μ l
Setup	R2_IndexX (10 μ M)	1.25 μ l
	R2_reverse (10 μ M)	1.25 μ l
	100 mM dNTPs	0.5 μ l
	Buffer	10 μ l
	DMSO	2.5 μ l
	Herculase	0.5 μ l
	H ₂ O	21.5 μ l
<hr/>		
Thermocycling condition	95 °C	2 min
	95 °C	15 s
	60 °C	20 s
	72 °C	30 s
	72 °C	x12
	4 °C	3 min
		hold

10. PCR amplification can be verified by running a small amount (*i.e.*, 5 μ l) of the PCR2 reactions on a 2% analytical TBE gel (see Figure 2: 230 bp product).

Note: Carried-through PCR1 product (445 bp product) may remain visible but should not affect downstream pooling and HTS as most of this product is removed during the Library Clean Up (Procedure I). Carried-through product could affect accurate quantification but does not contain sequences necessary for HTS and, therefore, do not interfere with sequencing (Procedure K).

11. Combine together all PCR2 reactions to create a vRNA PCR2 Pool.

12. Freeze the vRNA PCR2 Pool at -20 °C or at 80 °C.

I. Library Clean-Up: Double-Sided SPRI

Individual libraries are cleaned up prior to sequencing in order to remove excess primer and other nonspecific products (including PCR1 product that may be carried through). Double-sided Solid

Phase Reversible Immobilization (SPRI) accomplishes this but other approaches, such as Gel Purification can also be used. The bead:DNA ratio used here results in sufficient isolation of the 230 bp amplicons used in sequencing, but other ratios may also be appropriate.

1. Vortex an appropriate volume of AMPureXP beads thoroughly and bring them to room temperature.
2. Mix PCR2 Pool with the beads at a 0.8x ratio (e.g., 40 µl of DNA for 32 µl of beads) and vortex for 5 s.
3. Incubate for 5 min at room temperature.
4. Place tubes on a magnetic rack and incubate for 5 min.
5. Transfer the supernatant to a new tube, without disturbing the beads.
6. Incubate the supernatant from the previous step with beads at a 1.5x ratio (e.g., 50 µl of supernatant with 75 µl of beads). Vortex for 5 s and incubate for 5 min at room temperature.
7. Place tube on the magnetic rack for 5 min and carefully remove and discard the supernatant by pipetting, without disturbing the beads.
8. Add 200 µl of 80% ethanol (make fresh), keeping the tube on the magnetic stand. Incubate for 30 s and remove and discard the supernatant.
9. Optional: Repeat Step 17 one more time.
10. Air dry the beads on the magnet for 5 min but no longer (to avoid overdrying the DNA/beads mixture).
11. Remove the tube from the magnet and resuspend the beads in 45 µl of nuclease-free H₂O.
12. Vortex DNA/Bead mix for 5 s and incubate at room temperature for 2 min.
13. Place on the magnetic rack for 3 min. Carefully transfer 35 µl of supernatant to a new tube without carrying over beads. The supernatant should appear clear; if not, repeat this step to get rid of leftover beads.
14. Store cleaned-up libraries at -20 °C or -80 °C (or for at least several weeks at 4 °C).

J. Quantification of Libraries

The amplicon libraries need to be precisely quantified prior to pooling and sequencing to maximize sequencing output of adapter- and sgRNA-containing amplicon DNA during High-Throughput Sequencing (HTS) (Procedure L). Accurate quantification of library DNA is done with the Qubit dsDNA HS Kit and will be enhanced by calibrating the fluorometer with the provided calibration standards each time the assay is performed. Qubit dsDNA HS Assay Kit Manufacturer's protocol: assets.thermofisher.com/TFS-Articles/LSG/manuals/Qubit_dsdNA_HS_Assay_UG.pdf.

1. Quantify the dsDNA present in each cleaned-up PCR2 library sample, following the instructions for the Qubit dsDNA HS Kit as follows:
 - a. Prepare the Qubit solution by diluting Qubit reagent 1:200 in the provided buffer.
 - b. For each sample, add 2 µl of sample to 198 µl of the diluted buffer.
 - c. Read out quantification of dsDNA in each sample on the Qubit and record.

K. Pooling libraries for High-Throughput Sequencing

In this step, the amplicon libraries previously prepared are sequenced by High-Throughput Sequencing (HTS). Libraries are typically sequenced using Illumina miSeq or HiSeq, but other HTS methods may also be appropriate.

1. Calculate the concentration of all cleaned-up libraries and convert to nM.
For example, for an amplicon size of 230 bp, 1 ng/ μ l equates to a concentration of 6.59 nM.
2. Dilute the libraries to a final concentration of 2 nM in nuclease-free H₂O or other appropriate dilution for your sequencing method.
3. Pool together individual 2 nM libraries in a single tube to create a final 2 nM Pool for sequencing.

L. High-Throughput Sequencing (HTS) of HIV-CRISPR Libraries

In this step, the amplicon libraries previously amplified, cleaned-up and quantified are sequenced by High-Throughput Sequencing (HTS). An Illumina miSeq or HiSeq can be used, but other HTS methods may also be appropriate.

Notes:

- a. *The typical output of a miSeq run is usually 20% that of a HiSeq Rapid Run Lane. However, depending on the screen, a miSeq can be adequate. The sequencing depth required will depend on a number of factors, including the precise experimental setup, strength of selection and efficiency of nucleic acid extraction/amplification/clean-up. Typically, we obtain 1-5 million reads per sample, which is more than sufficient. More information on how to determine appropriate sequencing depth can be found on the manufacturer's website: illumina.com/science/technology/next-generation-sequencing/plan-experiments/coverage.html.*
- b. *Adding PhiX DNA may be necessary to avoid sequence quality issues caused by the low diversity samples, a common problem in amplicon sequencing. A PhiX spike of at least 20% is a useful starting point and can be optimized further in order to gain additional sequencing depth of the samples. Because 50 cycles are sufficient for HIV-CRISPR sequencing with the current amplification protocol/primer sets, we use the HiSeq or miSeq on a Single-Read 50 (SR50) setting.*

M. HIV-CRISPR Screen Analysis

A variety of data analysis methods can be used to analyze CRISPR screen data. The goal of this protocol is to identify genes affecting HIV replication: therefore, our data analysis approach aims at calculating which gene knock-out leads to increased or reduced HIV infection. To do this, we calculate the relative frequency of each sgRNA sequence in HIV-CRISPR genomes packaged in virions as compared to sgRNA sequences encoded in HIV-CRISPR genomes integrated in cells. The assumption is that sgRNAs targeting antiviral factors will be enriched in the virions and sgRNAs targeting cofactors that are required for or that enhance infection will be depleted in the viral fraction. Statistical methods explained below allow to take into account all the sgRNAs targeting a single gene to assign a score to each gene.

An example analysis pipeline: Raw BCL files are converted to FASTQ files using the bcl2fastq conversion software from Illumina. FASTQ files are then demultiplexed to assign reads to the different samples, using FASTX Toolkit Barcode Splitter. For each sample, reads are then trimmed to the 20 bp variant sgRNA sequence and aligned to the sgRNA library using Bowtie (Langmead et al., 2009). As a negative control, NTC (Non-Targeting Control) sgRNA sequences can be iteratively binned to create an NTC sgRNA gene set as large as the set of genes in the sgRNA library itself. Relative enrichment or depletion of sgRNAs and gene scores are determined with the MAGeCK statistical package using default parameters (Li et al., 2014). MAGeCK takes into account the enrichment of all sgRNAs for a gene across replicates to assign a MAGeCK Gene Score for each gene. Examples of analyzed datasets can be found in our original publication (OhAinle et al., 2018).

Note: Replicate CRISPR/Cas9 screens tend to generate reproducible results (r^2 values of > 0.9 for gDNA replicates and > 0.8 for vRNA replicates can be achieved). The most robust hits are those hits that are conserved across experiments. Variability in the results can be due to a lack of sampling of the library at various steps in the process: adequate infection of cells, efficient nucleic acid extraction, sequencing depth (i.e., insufficient read depth), or other experimental variables. The correlation between replicates, the degree of enrichment of Non-Targeting Control (NTC) sgRNAs, the overall MAGeCK Gene Scores, p-value and False Discovery Rate (FDR) of top scoring hits (as compared to the “NTC gene set”) are good indicators of the quality of the data generated. However, any hits identified must be validated to determine a true cutoff of signal to noise (i.e., empirically-derived). To avoid the risk of contamination, fresh kits and reagents are used where possible.

Recipes

1. 10x Tris/Borate/EDTA (TBE) running buffer (500 ml)
54 g of Tris base
27.5 g of boric acid
20 ml of 0.5 M EDTA
Adjust the pH to 8.3 by adding HCl
Dilute to 1x in water prior to electrophoresis
2. 20% sucrose solution (200 ml)
40 g sucrose
400 µl 0.5 M EDTA
4 ml 1 M HEPES
5 ml 4 M NaCl
Filter using Steriflip 50 ml conical tubes and keep sterile at 4 °C for up to a year
The EDTA stock is kept at 4 °C

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Competing interests

The authors declare no competing interests.

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Screening Method for CRISPR/Cas9 Inhibition of a Human DNA Virus: Herpes Simplex Virus

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[Abstract] The efficiency of cleavage of individual CRISPR/Cas9-sgRNAs remains difficult to predict based on the CRISPR target sequence alone. Different intracellular environments (dependent on cell type or cell cycle state for example) may affect sgRNA efficiency by altering accessibility of genomic DNA through DNA modifications such as epigenetic marks and DNA-binding proteins (e.g., histones) as well as alteration of the chromatin state of genomic DNA within the nucleus.

We recently reported a multi-step screening method for the identification of efficient sgRNAs targeting the Herpes simplex virus (HSV-1) genome and reported a differential mechanism for viral inhibition by CRISPR-Cas9 in the latent versus lytic phase. The screening platform detailed in this protocol allows step-by-step testing of the efficiency of cleavage in a cell-free system and in the context of viral target cells such as human foreskin fibroblasts followed by functional testing of the effects of CRISPR/sgRNA on viral protein expression, replication, and reactivation. This strategy could be readily applied to other target cells such as pluripotent stem cell-derived human sensory neurons or other human DNA viruses.

Keywords: Herpes simplex virus, HSV, *Herpesviridae*, CRISPR, CRISPR/Cas9, HSV keratitis, Recurrent genital herpes, HSV encephalitis

[Background] Herpes simplex virus (HSV) is a neurotropic DNA virus of the *Herpesviridae* family that causes lifelong and incurable infection in the majority of the human population and can lead to significant morbidity and mortality (Liesegang et al., 1989; Roizman et al., 2013). After the initial acute (primary) infection, HSV establishes lifelong latent infection within sensory ganglia. Latent HSV can reactivate periodically and cause serious illness such as HSV keratitis, the most common cause of corneal blindness (Liesegang et al., 1989), and recurrent genital herpes, which carries high morbidity. In the fetus, neonate and the immunosuppressed, herpesviruses such as HSV-1 and -2, human cytomegalovirus (HCMV), and Varicella-Zoster Virus (VZV) can cause disseminated disease with high mortality including meningitis and encephalitis. In the US, HSV infection occurs in 1 in 3,200 neonates (Thompson and Whitley, 2011), and half of these infants have disseminated disease or encephalitis

(Kimberlin, 2004), which, despite antiviral treatment, carries 25% mortality and long-term neurologic morbidity in more than two-thirds of survivors (Kimberlin et al., 2001). In addition, HSV1 infection has been linked with Alzheimer's disease risk in APOE-E4 carriers (Itzhaki, 2018).

Current antiviral treatments (nucleoside analogues) do not prevent establishment of latency or episodes of reactivation and are not effective for clearing latent HSV from infected individuals. Thus, there is a critical need for novel, specific, and efficacious therapeutic and prophylactic avenues that directly cleave and/or edit viral genomes and permanently eliminate latent virus or disable its reactivation and effectively suppress viral replication in disseminated acute HSV infections.

CRISPR/Cas9 systems have evolved in bacteria as a form of adaptive immunity to provide resistance against bacteriophage infection and plasmid transformation (Bhaya et al., 2011; Doudna and Charpentier, 2014; Gaj et al., 2013; Hsu et al., 2014). This system has been successfully transported into human cells (Cong et al., 2013; Mali et al., 2013). However, sgRNAs with comparable cutting efficiencies in a cell-free system can differ significantly when used in living cells (Oh et al., 2019), indicating that the intracellular environment may alter the accessibility of genomic DNA for cleavage by CRISPR/Cas9. Possible mechanisms are the covalent modification of genomic DNA in the form of epigenetic marks, differential density of DNA binding proteins (such as histones), and alteration of the chromatin state of genomic DNA in different cell types and/or cellular states (Yarrington et al., 2018; Liu et al., 2020).

The screening platform described in this protocol allows identification of sgRNAs that efficiently cleave the HSV genome and inhibit lytic viral replications as well as reactivation of quiescent virus in the chosen type of target cells. We used SaCas9 to allow for further testing of the antiviral activity of individual SaCas9/sgRNAs *in vivo* using recombinant AAV-based delivery systems. This screening strategy can be extended to other HSV target cells such as human sensory neurons which can be differentiated from human pluripotent stem cells (Chambers et al., 2012; Young et al., 2014). In addition, this screening strategy can be applied to clinically important members of the *Herpesviridae* family such as HSV-2, HCMV, VZV and KSHV as well as other human DNA viruses lacking effective therapies.

Materials and Reagents

1. Eppendorf Safe-Lock Tubes, 1.5 ml (Thermo Fisher Scientific, Eppendorf, catalog number: 022363212)
2. PCR tubes (any kind)
3. Pipette tips 10 µl, 200 µl, 1,000 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: 2140-05, 2160P, 2079)
4. Tissue culture flasks T-75 filtered flasks (Corning, Falcon®, catalog number: 353824)
5. 50 ml Conical tube (Corning, Falcon®, catalog number: 352098)
6. 0.45 µm syringe filter (Pall, catalog number: 4508)
7. 6-well plates (Corning, catalog number: CLS3516)
8. AmpliTaq Gold™ 360 Master Mix (Thermo Fisher Scientific, Applied Biosystems, 4398901)

9. Wizard® SV Gel and PCR Clean-Up System (Promega, catalog number: A9281)
10. Platinum™ II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, Invitrogen, catalog number: 14966001)
11. MAXIscript™ T7 Transcription Kit (Thermo Fisher Scientific, Invitrogen, catalog number: AM1314)
12. Fusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, New England Biolabs, catalog number: M0530S)
13. Gene Snipper™ SaCas9 Protein (CRISPR-associated endonuclease Cas9 from *Staphylococcus aureus*) with SaCas9 Buffer: 200 mM HEPES, 50 mM MgCl₂, 1 M NaCl, 1 mM EDTA, pH 6.5 (Biovision, catalog number: M1280) or SpCas9 Protein (CRISPR-associated endonuclease Cas9 from *S. pyogenes*) with 1x NEBuffer 3.1: 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, pH 7.9 (New England Biolabs, catalog number: M0386)
14. E.Z.N.A.® Micro RNA Kit (Omega Bio-Tek, catalog number: R7034-01)
15. QIAfilter Plasmid Midi Kit (Qiagen, catalog number: 12245)
16. Lenti-X qRT-PCR Titration Kit (Takara, catalog number: 631235)
17. BsmBI restriction enzyme (Thermo Fisher Scientific, New England Biolabs, catalog number: R0580)
18. DNeasy Blood & Tissue kit (Qiagen, catalog number: 69504)
19. Agarose (Sigma-Aldrich, catalog number: A9539)
20. Polyethyleneimine (Polysciences, catalog number: 23966, average Mw ~25,000)
21. Effectene (Qiagen, catalog number: 301425)
22. NuPAGE™ LDS Sample Buffer (4x) (Thermo Fisher Scientific, Invitrogen, catalog number: NP0008)
23. NuPAGE 4-12% Bis-Tris Gels (Thermo Fisher Scientific, Invitrogen, catalog number: NP0321PK2)
24. NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific, Invitrogen, catalog number: NP0001)
25. Nitrocellulose membrane (Bio-Rad, catalog number: 1620112)
26. Odyssey Blocking Buffer (TBS) (LI-COR, catalog number: 927-50000)
27. IRDye 800CW (P/N: 926-32211) or IRDye 680RD (P/N: 926-68070) secondary antibody (LI-COR)
28. Giemsa stain (Sigma-Aldrich, catalog number: G4507 or GS500)
29. Assay buffer (see Recipes)
30. KCl
31. MgCl₂
32. Tris
33. Glycerol
34. EDTA
35. SDS

36. Bromophenol blue
37. Reaction Stop Buffer (see Recipes)

Media

1. DMEM (Thermo Fisher Scientific, Gibco, catalog number: 11960051)
2. Opti-MEM™ I (Thermo Fisher Scientific, Gibco, catalog number: 31985062)
3. Fetal Bovine Serum (Thermo Fisher Scientific, Gibco, catalog number: 26140079)
4. Bovine calf serum (Thermo Fisher Scientific, catalog number: 16030074)
5. L-Glutamine (Thermo Fisher Scientific, Gibco, catalog number: 25030164)
6. G418 Geneticin (Thermo Fisher Scientific, Gibco, catalog number: 10131035)
7. Puromycin Dihydrochloride (Thermo Fisher Scientific, Gibco, catalog number: A1113802 or Santa Cruz, catalog number: sc-134220)
8. Polybrene Infection reagent (Millipore Sigma, catalog number: TR-1003-G or Santa Cruz: sc-134220)
9. GAMMAGARD LIQUID Immune Globulin Infusion (Takeda, Shire, catalog number: LE1500190, NDC: 0944 2700 02)

Plasmids

1. lenti-SaCas9-sgRNA-Puro

Can be obtained by cloning SaCas9 and its trans-activating sgRNA into the lentiCRISPRv2 plasmid (addgene #52961). lentiCRISPRv2 is digested using BsmBI (NEB) and BarnHI (NEB) and purified using a DNA purification kit (Zymo). DNA assembly can be performed using NEBuilder® HiFi DNA Assembly (NEB) according to the manufacturer's protocol with three DNA fragments, the purified linear lentiCRISPRv2, a double-stranded DNA gBlock (IDT) containing the sgRNA sequence (IDT, 5' CTTTATATATCTTGAGAAAGGA CGAACACCCGGAGACGtGATATCaCGTCTCAGTTTAGTACTCTGGAAACAGAAATCTACTAA AACAAAGGCAAAATGCCGTGTTATCTCGTCAACTGTTGGCGAGATTTTGAATTCTGTAGA CTCGAGGCCTTGACAT TG 3') and a PCR fragment containing SaCas9.

2. psPAX2 (Addgene plasmid #12260)
3. pVSV-G (Addgene plasmid # 8454)

Cell lines and media

Human Foreskin Fibroblasts (HFF, Hs27, ATCC, catalog number: CRL-1634), U2OS (known as U-2 OS, ATCC, catalog number: HTB-96), 293T (ATCC, catalog number: CRL-3216) and Vero (ATCC, catalog number: CCL-81) cells can be obtained from the American Type Culture Collection.

1. HFF and 293T cells: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS)
2. V27 cells: DMEM supplemented with 5% (v/v) FBS, 5% (v/v) bovine calf serum (BCS), 2 mM L-glutamine, and 500 µg/ml of G418 in 5% CO₂ (Rice *et al.*, 1989)

3. U2OS and Vero cells: DMEM supplemented with 5% (v/v) FBS, 5% (v/v) bovine calf serum (BCS), and 2 mM L-glutamine in 5% CO₂
4. FO6 cells: are maintained in DMEM supplemented with 5% (v/v) FBS, 5% (v/v) BCS, and 2 mM L-glutamine with 500 µg/ml of G418 (Gibco) and 300 µg/ml of hygromycin B (Invitrogen) in 5% CO₂ (Samaniego et al., 1997)

Viruses

The KOS strain is a commonly used wild-type HSV-1 strain (Schaffer et al., 1970). HSV-1 d109 is a KOS strain-derived mutant that is deleted for all five immediate early (IE) genes and contains the green fluorescent protein (GFP) gene under the control of the human cytomegalovirus (HCMV) IE promoter in the HSV-1 *UL54* gene locus. d109 can be grown and titrated on U2OS ICP4/27 and FO6 cells (Samaniego et al., 1998).

MiSeq adapter primers (Table 1)

Forward adapter primer: 5'-TCTTCCCTACACGACGCTCTCCGATCT-CRISPR site forward primer-3'

Reverse adapter primer: 5'-TGGAGTTCAGACGTGTGCTCTCCGATCT-CRISPR site reverse primer-3'

HSV-1 gene specific primers

UL30-5_for: 5'-CCCAAGGTGTACTGCGGG-3'

UL30-5_rev: 5'-CTCCACGTTCTCCAGGATGT-3'

UL29-3_for: 5'-ATAGACTCGAGGCCAGGG-3'

UL29-3_rev: 5'-TGACGAAAACCACGAGGGC-3'

Table 1. MiSeq barcode primers

Primer ID	Barcode Sequence	Primer sequence
BARCODE_FOR	n/a	aatgatacggcgaccaccgagatctacactttccctacacgacgt
BARCODE_REV_1	ATCACGA	caagcagaagacggcatacgagatTCGTGATgtactggagttcagacgtgtgt
BARCODE_REV_2	CGATGTA	caagcagaagacggcatacgagatTACATCGgtactggagttcagacgtgtgt
BARCODE_REV_3	TTAGGCA	caagcagaagacggcatacgagatTGCCTAAgtactggagttcagacgtgtgt
BARCODE_REV_4	TGACCAA	caagcagaagacggcatacgagatTTGGTCAGtactggagttcagacgtgtgt
BARCODE_REV_5	ACAGTGA	caagcagaagacggcatacgagatTCACTGTgtactggagttcagacgtgtGCT
BARCODE_REV_6	GCCAATA	caagcagaagacggcatacgagatTATTGGCgtactggagttcagacgtgtGCT
BARCODE_REV_7	CAGATCA	caagcagaagacggcatacgagatTGATCTGgtactggagttcagacgtgtgt
BARCODE_REV_8	ACTTGAA	caagcagaagacggcatacgagatTCAAGTgtactggagttcagacgtgtgt
BARCODE_REV_9	GATCAGA	caagcagaagacggcatacgagatTCTGATCgtactggagttcagacgtgtgt
BARCODE_REV_10	TAGCTTA	caagcagaagacggcatacgagatTAAGCTAgtactggagttcagacgtgtgt
BARCODE_REV_11	GGCTACA	caagcagaagacggcatacgagatTGTAGCCgtactggagttcagacgtgtgt

Table 1. Continued

BARCODE_REV_12	CTTGTA	caagcagaagacggatacgagatTTACAAGgtgactggagttcagacgtgtct
BARCODE_REV_13	AGTCAAC	caagcagaagacggatacgagatGTTGACTgtgactggagttcagacgtgtct
BARCODE_REV_14	AGTTCCG	caagcagaagacggatacgagatCGGAACtgtgactggagttcagacgtgtct
BARCODE_REV_15	ATGTCAG	caagcagaagacggatacgagatCTGACATgtgactggagttcagacgtgtct
BARCODE_REV_16	CCGTCCC	caagcagaagacggatacgagatGGGACGGgtgactggagttcagacgtgtct
BARCODE_REV_17	GTCCGCA	caagcagaagacggatacgagatTGC GGACgtgactggagttcagacgtgtct
BARCODE_REV_18	GTGAAAC	caagcagaagacggatacgagatGTTT CACgtgactggagttcagacgtgtct
BARCODE_REV_19	GTGGCCT	caagcagaagacggatacgagatAGGCCACgtgactggagttcagacgtgtct
BARCODE_REV_20	GTTCGG	caagcagaagacggatacgagatCCGAAACgtgactggagttcagacgtgtct
BARCODE_REV_21	CGTACGT	caagcagaagacggatacgagatACGTACGgtgactggagttcagacgtgtct
BARCODE_REV_22	GAGTGG	caagcagaagacggatacgagatTCCACTCgtgactggagttcagacgtgtct
BARCODE_REV_23	ACTGATA	caagcagaagacggatacgagatTATCAGTgtgactggagttcagacgtgtct
BARCODE_REV_24	ATTCCCTT	caagcagaagacggatacgagatAAGGAATgtgactggagttcagacgtgtct

Equipment

1. Pipettes (capable of accurately pipetting from 1 µl-10 ml) (any source)
2. Thermocycler (Bio-Rad, model: C1000)
3. Vortex (any source)
4. Micro centrifuge capable of 14 kG (Eppendorf)
5. Incubators (37 °C)
6. DNA gel electrophoresis apparatus
7. Odyssey CLx Imaging System (LI-COR)
8. UV transilluminator (or handheld UV lamp)
9. Spectrophotometer (Thermo Fisher Scientific, model: NanoDrop 2000)

Software

1. Outknocker (<http://www.outknocker.org/>)
2. Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>)
3. mutect2 (<https://gatk.broadinstitute.org/hc/en-us/articles/360037593851-Mutect2>)
4. Prism 6 (Version 6.01) software

Procedure

- A. Cell-free CRISPR/Cas9 cutting assay
 1. Design sgRNAs
 - a. Identify essential genes (e.g., *UL29*, *UL30*, *UL54*, *a4* for HSV-1) or DNA regions within the viral genome.

- b. Design appropriate sgRNA target sequences within ~1 kbp downstream of the start codon. The requirement for SaCas9 cleavage is the presence of a PAM sequence (5'-NNGRRT-3') immediately downstream of the ~21 base pair target. Select multiple independent CRISPR sgRNAs for each viral gene target for efficiency testing.
 - c. Assess off-target activity of sgRNAs: The sgRNA sequences should be unique to the viral genome to prevent off-target cleavage of human DNA. Useful web sites for sgRNA identification and ranking of off-target activity are <http://chopchop.cbu.uib.no/> [CHOP CHOPv2 (Labun et al., 2016) and crispr.mit.edu (Zhang lab, MIT) (Hsu et al., 2013)].
2. *In vitro* sgRNA synthesis
- a. Generate templates for T7 *In vitro* transcription of sgRNAs
 - i. Design and order oligos for sgRNA synthesis: For each sgRNA design a 60 nt forward oligo (target specific oligo), containing the T7 promoter (5'-TAATACGACTCACTATA) followed by 21 nt of the specific SaCas9 sgRNA DNA binding sequence with the first 2 nucleotides replaced by GG (GGNNNNNNNNNNNNNNNNNNNN) for T7 initiation and a constant 23 nt tail of sgRNA scaffold sequence for annealing (GTTTTAGTACTCTGGAAACAGAA-3'). These forward oligos are used in combination with a constant 82 nt reverse oligo (5'-AAAAAATCTGCCAACAGTTGACGAGATAAACACGGCATTTCGCCTTGTTAGTAGATTCTGTTCCAGAGTACTAAAAC-3') encoding the reverse-complement of the tracrRNA tail to add the sgRNA invariable 3' end (SaCas9 scaffold oligo, Figure 1).
 - ii. Anneal sgRNA primers with the tail primer: For each sgRNA assemble the following reaction in PCR tubes:

Reagent	[μl]
Oligo 1 (variable sgRNA oligo), 100 μM	1
Oligo 2 (constant tail oligo), 100 μM	1
H ₂ O	8
Total	10

Anneal protocol:

Hold at 95 °C for 5 min.

Ramp from 95 °C → 85 °C at 2 °C/s

Ramp from 85 °C → 25 °C at 0.1 °C/s

Hold at 4 °C.

- iii. Fill in single-stranded DNA (ssDNA) overhangs to generate double- stranded DNA (dsDNA) templates for T7 transcription by assembling the following reaction for each sgRNA in PCR tubes (Figure 1):

Reagent	[μl]
AmpliTaq Gold™ 360 Master Mix	10

Taq polymerase (5 U)	1
Anneal Rx from ii.	9
Total	20

Incubate at 72 °C for a total of 1.5 h and add an additional 1 µl Taq after 45 min.

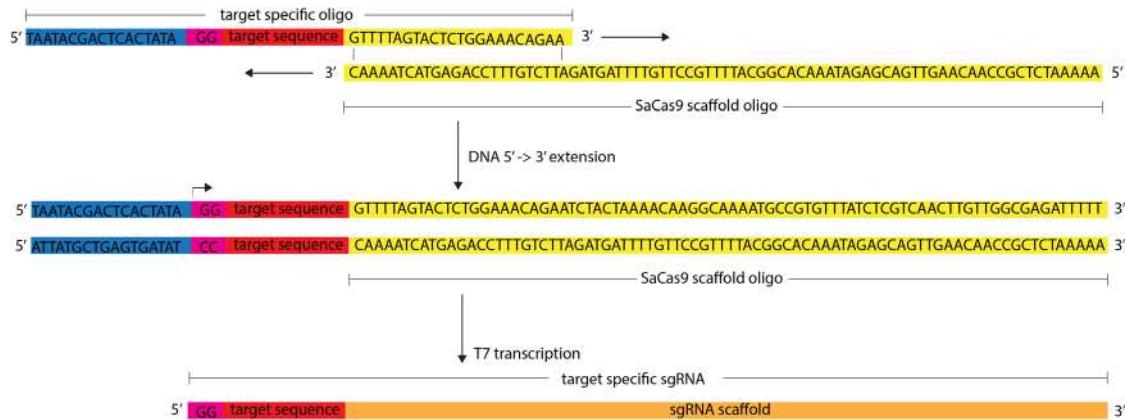


Figure 1. T7 *in vitro* transcription of sgRNAs. The 60 nt target-specific oligo contains the T7 promoter sequence followed by 21 nt of the specific *S. aureus* Cas9 sgRNA DNA binding sequence with the first 2 nucleotides replaced by GG and a 23 nucleotide overlap sequence of the SaCas9 scaffold oligo. The overlap sequence is complementary to the first 23 nt of the (constant) SaCas9 scaffold oligo. The two oligos are annealed and both oligos extended from their 3' ends by DNA polymerase resulting in a double-stranded DNA template for T7 transcription. RNA polymerase recognizes the double-stranded DNA of the T7 promoter and initiates transcription at the 'GG'. The resulting sgRNA contains the target-specific CRISPR sequence as well as the RNA scaffold.

- iv. Purify the dsDNA using the Promega Wizard Clean up kit and elute in 20 µl H₂O.
 - v. (Optional) Run dsDNA templates on an agarose gel to check the size.
- b. sgRNA *in vitro* transcription (IVT):
- i. For each sgRNA assemble the following reaction using the MAXIscript™ T7 Transcription Kit and approximately 150 ng of dsDNA template and incubate overnight at 37 °C:

Reagent	[µl]
ATP solution	2
CTP solution	2
GTP solution	2
UTP solution	2
10X Reaction Buffer	2
Template DNA (150 ng)	~4
T7 Enzyme Mix	2

H ₂ O	4
Total	20

Note: Assemble transcription reaction at room temp (The spermidine in the 10x Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice). Add the 10x Reaction Buffer after the water and the ribonucleotides are already in the tube

- ii. Remove template DNA: Add 56 µl H₂O and 4µl DNase turbo to each IVT reaction and incubate at 37 °C for 15 min.
 - iii. sgRNA purification: Purify the sgRNAs using the Omega EZNA PF kit and measure the concentration of the purified sgRNAs using a NanoDrop 2000. Elute in H₂O or assay buffer). After purification, dilute the sgRNAs to 200 ng/µl each and store at -80 °C.
 - iv. (Optional) Confirm the expected size (~97bp) of the sgRNA on an RNA gel.
3. Generate dsDNA substrate for the *In vitro* cutting assay
 - a. For each sgRNA amplify a 500-2,000 bp segment around the sgRNA sequence to be tested from HSV-1 genomic DNA using PCR. If the amplification is difficult, dsDNA for the cleavage assay can also be obtained as g-blocks (IDT).
 - b. Purify the PCR product using the Promega Wizard Clean up kit.
 4. *In vitro* cutting assay with CRISPR sgRNA and SaCas9 protein
 - a. Reagent setup:
 - i. Dilute Cas9 protein in assay buffer to 500 nM.
 - ii. Dilute purified PCR product to 50-100 ng/µl in H₂O.
 - iii. Confirm each sgRNA is at 200 ng/µl or higher.
 - b. Assembly of cleavage assay: Each guide requires four wells of a PCR strip. Cleavage assays are performed in a reaction volume of 10 µl with final SaCas9 concentrations of 300 nM, 100 nM, 33 nM, and 0 nM, 400ng sgRNA, 200 ng dsDNA substrate in 1x SaCas9 nuclease reaction buffer.
 - i. Add 6 µl of assay buffer to wells 2-4 of a PCR strip and 6 µl of 500 nM SaCas9 protein to well 1. Then add 2 µl of 500 nM SaCas9 protein to well 2, mix, transfer 2 µl of this to well 3, mix and remove and discard 2 µl of this mix. Since the final volume of the reaction is 10 µl, the final Cas9 concentrations are 300 nM, 100 nM, 33 nM, and 0 nM.
 - ii. Add 2 µl of sgRNA to each of the 4 wells.
 - iii. Incubate for 10 min at 37 °C to allow SaCas9-sgRNA complexes to form.
 - iv. Add 2 µl purified PCR product to each of the 4 wells.
 - v. Incubate for 30 min at 37 °C.
 - vi. Add 4 µl of reaction stop buffer to each well and incubate at 80 °C for 10 min.
 - vii. Run all 10 µl of all reactions on a 2% agarose gel and image.
 - viii. Analysis: Because the assay provides sgRNA in excess, SaCas9 protein is the limiting factor. Cutting efficiency is constrained by the total amount of template provided, but in

general, sgRNAs that work well show robust cutting (larger band at 0-50% intensity of no SaCas9 control) and there will be evidence of graded activity at lower SaCas9 concentrations. Figure 2 shows the results of the *in vitro* cleavage assay for three sgRNA targeting the essential HSV *UL30* gene (encoding the viral polymerase).

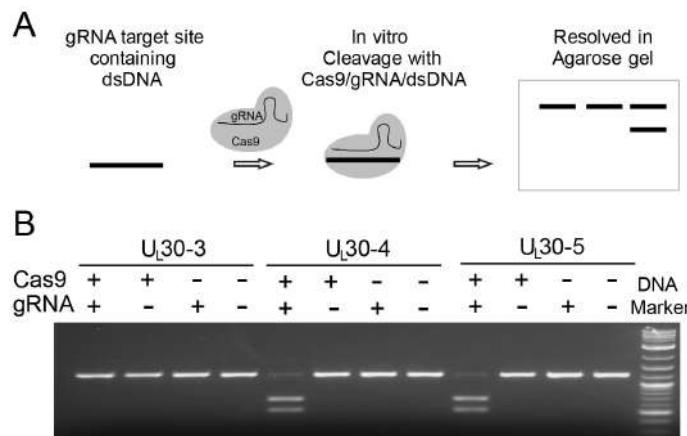


Figure 2. *In vitro* cleavage assay for the essential HSV-1 gene *UL30* (Oh et al., 2019). A. Schematic diagram of *in vitro* cleavage assay; B. Results are shown for three sgRNAs targeting *UL30* (*UL30-3*, *-4*, and *-5*). T7 *in vitro* transcribed sgRNA was combined with SpCas9 protein and a PCR template containing the CRISPR sequence, incubated 1 h at 37 °C and run on an agarose gel. Lane (1) SpCas9+sgRNA, lane (2) Cas9 only, lane (3) sgRNA only, lane (4) no Cas9/sgRNA. Efficient cutting is seen for *UL30-4* and *-5* but not *UL30-3*.

B. Cell-based CRISPR/SaCas9 cutting assay using next generation sequencing (MiSeq)

1. Cloning of sgRNAs into SaCas9 lentivirus expression vector
 - a. Clone the sgRNA CRISPR sequence into lentiCRISPR SaCas9 after *BsmBI* digestion using oligos as described previously (Sanjana et al., 2014; Shalem et al., 2014) to generate a lenti vector expressing SaCas9 and a sgRNA as well as the selection marker puro.
 - b. Purify plasmid DNAs using the endotoxin-free Midiprep kit (Qiagen) according to the manufacturer's protocol.
2. Lentivirus prep
 - a. Plate 3 × 10⁶ human 293T cells in a 100 mm dish 20-24 h before transfection.
 - b. Transfect the 293T cells with lenti-SaCas9-sgRNA-Puro (5 µg), psPAX2 (4 µg), and pVSVG (1 µg) using Effectene according to the manufacturer's protocol or using polyethyleneimine (PEI).
 - c. For PEI transfection, mix a total of 10 µg of DNAs in 500 µl of Opti-MEMI (Gibco) and 30 µg of PEI in 500 µl of Opti-MEMI.
 - d. Mix DNA and PEI (total 1 ml) and incubate at ambient temperature for 20 min.

- e. Add mixture directly to 293T cells containing fresh 5 ml of DMEM (supplemented with 10% (vol/vol) FBS and 2 mM glutamine).
 - f. Incubate the cells at 37 °C for 8-12 h, then replace media with 10 ml of fresh DMEM supplemented with 10-30% (vol/vol) FBS and 2 mM glutamine, and continue incubation at 37 °C.
 - g. At every 12-24 h for 48-60 h, harvest media and replace with fresh DMEM (supplemented with 10-30% (vol/vol) FBS and 2 mM glutamine).
 - h. Pool the harvested media on ice, filter the pooled media using 0.45 µm syringe filter (Pall).
 - i. Optional: To concentrate, centrifuge the virus for 1.5 h at 25,000 rpm (SW28 rotor of Beckman, ~120,000 x g) at 4 °C.
 - j. Titration of lentivirus: use the Lenti-X qRT-PCR Titration Kit (Takarabio) or qPCR Lentivirus Complete Titration Kit (abm) according to the manufacturer's protocol.
 - k. Keep lentivirus at 0-4 °C until transduction of target cells (within one day) or freeze at -80 °C for long-term storage.
3. For quiescent HSV infection:
 - a. Establish quiescent HSV-1 infection in HFF cells:
 - i. Infect HFF cells with the HSV-1 mutant *d109* (which is missing all 5 immediate early genes) at an MOI of 10 in PBS containing 0.1% glucose (wt/vol) and 0.1% BCS (v/v) for 1 h with shaking at 37 °C (Ferenczy and DeLuca, 2011).
 - ii. Replace virus inoculum with DMEM supplemented with 10% (v/v) FBS and incubate at 37 °C for 7-10 days.
 - b. Transduction of HFFs containing quiescent HSV-1 viral genomes with SaCas9/sgRNA
 - i. Transduce the *d109* infected cells with 2 or 10 ml of lentivirus (or at an MOI of 5) containing 3-4 µg/ml of polybrene in 6-well plates or in T150 flasks respectively (optional: repeat the lentivirus transduction next day).
 - ii. The next day, replace the transduction medium with fresh medium and incubate cells for 2 days at 37 °C followed by puromycin treatment (1 µg/ml) for 7-10 days.
 - iii. Isolation of HSV genomic DNA: Harvest cells and isolate genomic DNA using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol.
 4. For lytic HSV infection:
 - a. Transduction of HFFs with SaCas9/sgRNA:
 - i. To transduce HFF cells with lentivirus for the lytic infection assay, plate HFF cells at a density of 2×10^5 /well in a T25 flask one day prior to transduction.
 - ii. Transduce with 2-5 ml of lentivirus (or at an MOI of 5) containing 3-4 µg/ml of polybrene (optional: repeat the lentivirus transduction next day).
 - iii. The next day, replace the transduction medium with fresh medium and incubate cells for 2 days at 37 °C followed by puromycin treatment (1 µg/ml) for 7-10 days.
 - b. Lytic infection with wild-type HSV-1
 - i. Infect the transduced cells with wild-type HSV-1 at an MOI of 0.1 or 5 in phosphate-

- buffered saline (PBS) containing 0.1% glucose (wt/vol), 0.1% BCS (v/v) for 1 h with shaking at 37 °C.
- ii. Replace virus inoculum with DMEM containing 1% BCS and incubate at 37 °C.
 - iii. Harvest cells at various time points for viral replication by qPCR and titration.
 - iv. Isolation of genomic and viral DNA: isolate genomic DNA using the DNeasy Blood & Tissue kit according to the manufacturer's protocol.
5. Deep sequencing (MiSeq): The process of deep sequencing involves an initial PCR to generate a ~200 bp product around the region of interest followed by a second PCR to add barcodes to the amplicons. Following this, each of the products is run on an agarose gel to determine the relative amount of each amplicon. Based on the brightness of the amplicon band on the gel, the PCR products of a particular loci are pooled such that each amplicon is ~equally represented in the final pool. The pooled product is then run on an agarose gel and gel-purified before being submitted for sequencing. With a set of 24 unique barcoding primer pairs a total of 24 sgRNAs can be analyzed in parallel with one deep sequencing sample.
- a. Adapter (first round) PCR with target gene-specific primers
 - i. Design a primer set generating a ~200 bp product around the CRISPR site and add the adapter sequences to forward and reverse primer (see Figure 3). The CRISPR site should be in the center 1/3 of the amplicon to obtain good sequencing across the targeted site. The adapter sequences are used in the second round (barcoding) PCR for primer annealing.

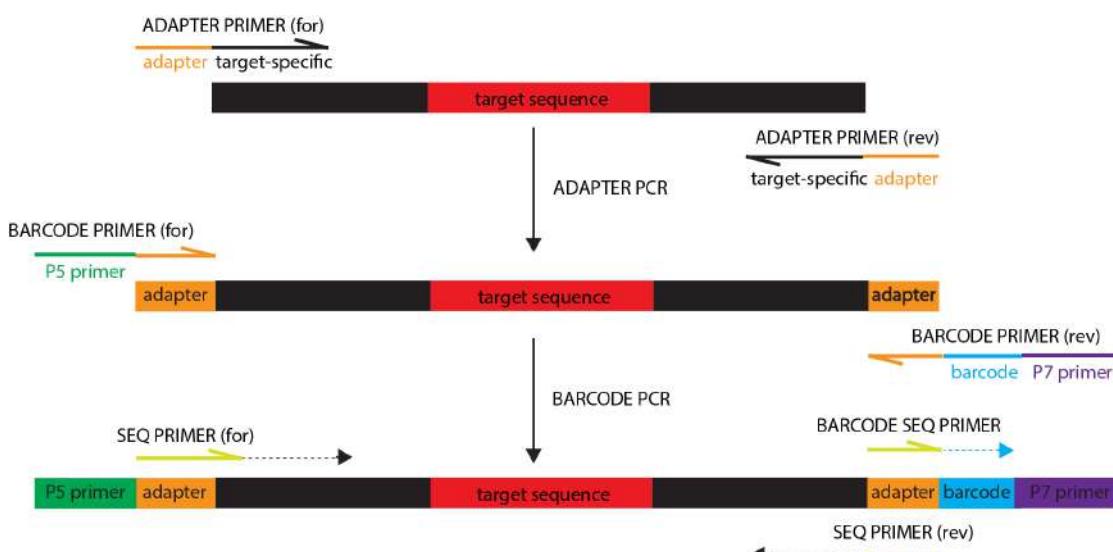


Figure 3. Deep sequencing (MiSeq) process. A diagram of the two step PCR (adapter PCR, barcode PCR). The first step (adapter PCR) uses gene specific primers (black) with extensions (orange) for the second step (barcode PCR). The following sequences are the adaptors that should be added to the gene-specific primers: Adapter sequence for forward primer: 5'-TCTTCCCTACACGACGCTCTCCGATCT-target specific sequence-3', Adapter sequence for reverse primer: 5'-TGGAGTTCAGACGTGTGCTCTCCGATCT-target specific sequence-3'.

The barcode PCR primers anneal to the adapter sequences and contain a barcode (blue) as well as P5/P7 sequences for binding to the flow cell. The three sequencing reads cover the amplicon/target sequence and read the barcode.

- ii. Perform a Phusion touch-down PCR, which increases the specificity of the reaction, according to the following protocol. Use the NEB calculator to determine the annealing temperature (<http://tmcalculator.neb.com/#!/>).

Reagent	[μ l]
5x GC buffer	2
dNTPs (10 mM)	0.2
Forward primer (25 μ M)	0.05
Reverse primer (25 μ M)	0.05
Template (~1-10 ng gDNA from B4b-iii)	1
Phusion polymerase	0.1
DMSO	0.3
H ₂ O	6.3
Total	10

Cycle protocol:

Initial denaturation 98 °C 30 s

15x

Denaturation 98 °C 10 s

Annealing 72 °C (-0.5 °C/cycle) 30 s

Elongation 72 °C 2 min

15x

Denaturation 98 °C 10 s

Annealing Tm 30 s

Elongation 72 °C 2 min

Final elongation 72 °C 5 min

Hold at 4 °C

- iii. Run a small amount (2 μ l) on a 1.5% agarose gel to ensure that the PCR worked. If so, move on to the second round (barcoding) PCR.

- b. Barcode (second round) PCR with barcoding primers: This PCR uses universal primers (not gene-specific, see Table 1) to attach the sequencing adaptors and barcode to the amplicon. The forward primer is the same, but there are 24 reverse primers containing different barcodes (for testing of up to 24 different sgRNAs in parallel).

- i. Set up a standard 10 μ l PCR mix using 1 μ l of the first round PCR product as the template as follows:

PCR Rx	[μ l]
5x GC buffer	2
dNTPs (10 mM)	0.2
Forward primer (10 μ M)	0.5
Reverse primer (5 μ M)	1
Adapter PCR (template)	1.0
Phusion	0.1
DMSO	0.3
H ₂ O	4.9
Total	10

Cycle protocol:

Initial denaturation 98 °C 30 s

20x

Denaturation 98 °C 10 s

Annealing 66 °C 20 s

Elongation 72 °C 20 s

Final elongation 72 °C 3 min

Hold at 4 °C

- ii. Run a small amount (2 μ l) on a 1.5% agarose gel. This allows you to ensure that the PCR worked and to normalize the amount of each amplicon when PCR products are pooled together for MiSeq. Of note, in some cases, one may see a large primer dimer amplicon around 175 bp in addition to the correct band.
- iii. Pool amplicons and perform gel extraction:
 - 1) Pool (up to 24) amplicons for different sgRNAs—do not pool across amplicons from different targeted viral genes as this will make the gel extraction more difficult. From the previous gel, estimate equal molarities when pooling (*i.e.*, compensate for weaker looking amplicons by adding more to the pool). This will even out the read counts across different amplicons during MiSeq.
 - 2) Run pooled amplicons on a 1.5% agarose gel and gel extract the DNA using the Promega Gel Extraction Kit.
- iv. Submit purified/pooled amplicons for IlluminaMiSeq using the 16S Metagenomic Sequencing Library Preparation kit (Illumina). Determine DNA concentrations using the Qubit fluorometer 2.0 (Life Technologies, USA) with the Qubit dsDNA High Specificity assay kit. Sequence DNA libraries by IlluminaMiSeq (150 bp paired-end [Gagnon *et al.*, 2014]).

6. Indel analysis: Determine indel frequencies for each sgRNA by quantifying aligned reads containing insertions or deletions 1bp or larger using (<http://www.outknocker.org/>). Analyze indel size using the ICE analysis toolbox (<https://www.synthego.com/products/bioinformatics/crispr-analysis>). Figure 4 shows the analysis of indel mutations in the HSV-1 genome during the quiescent infection for sgRNAs targeting the essential HSV-1 genes *UL29* and *UL30*.

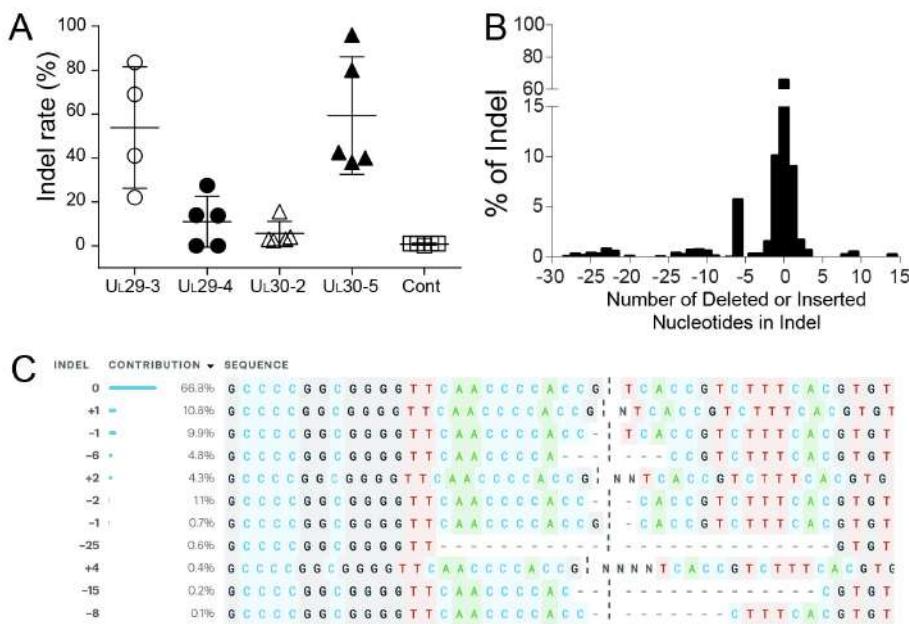


Figure 4. Indel mutations in the HSV-1 genome during the quiescent infection (Oh et al., 2019). A. Indel mutation frequencies of quiescent d109 genomes are shown at the indicated sgRNA target sites. B. Histogram representing the frequency (count) of indel lengths induced by SaCas9/UL30-5 in quiescent d109 genomes. (-) deletions (+) insertions. C. Examples of sequences that show mutations induced by SaCas9/UL30-5 in quiescent d109 viral genomes.

7. Analysis of off-target effects in the human an viral genome
- Transduce HFFs with lentivirus expressing SaCas9/sgRNA as above and SaCas9 without sgRNA as control.
 - Isolate genomic DNA using the DNeasy Blood & Tissue kit according to the manufacturer's protocol.
 - Perform 30x whole genome sequencing (WGS) of SaCas9/sgRNA treated sample and control.
 - To detect off-target effects in the human genome: Map WGS reads to the human genome (hg38) and use mutect2's tumor-normal calling treating the CRISPRed sample as a 'tumor' to identify possible variants within the human genome in the sgRNA treated HFFs relative to the control. Use Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>) in offline mode to look for all predicted off-target sites with 6 or fewer mismatches within hg38 for each sgRNA and determine the overlap between predicted variants and off-target sites.

- e. To detect off-target effects in the viral (HSV) genome: Align the unmapped reads to the HSV-1 KOS genomic sequence (GenBank: KT899744) and call variants using mutect2's tumor-normal calling. One can then determine the overlap between predicted variants and off-target sites within HSV-1 KOS.

C. Functional testing of CRISPR/SaCas9 antiviral activity (see Figure 5A)

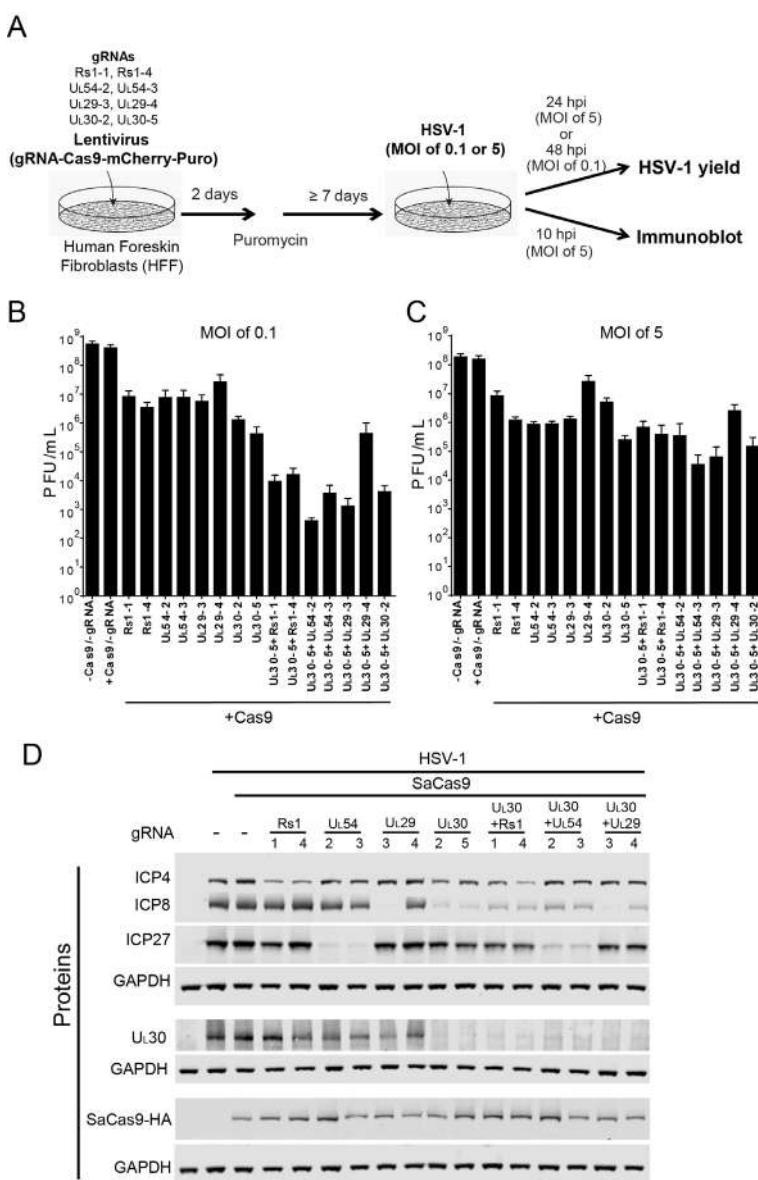


Figure 5. Effect of CRISPR-Cas9 on HSV-1 lytic infection (Oh et al., 2019). A. Experimental scheme of SaCas9/sgRNA-mediated inhibition of HSV lytic infection. B. and C. HFFs transduced with lentivirus expressing SaCas9 and sgRNAs were infected with HSV-1 at an MOI of 0.1 (C) or 5 (D) and harvested at 48 hpi or 24 hpi, respectively. Viral yields were determined by plaque assays. The histogram shows the mean values and standard deviations of biological replicates at an MOI of 0.1 (N = 3) or at an MOI of 5 (N = 4). All the sgRNA added conditions

showed statistical significance compared to +Cas9/-gRNA (*t*-test, **P* < 0.05). D. HFFs transduced with lentivirus expressing SaCas9 and sgRNA were infected with HSV-1 at a MOI of 5 and harvested at 10 hpi. Proteins were detected using immunoblotting with antibodies specific for the indicated proteins. Three SDS-PAGE gels loaded with the same amount of proteins were used to detect multiple proteins. Immunoblots of GAPDH were shown as a control under the individual immunoblots.

1. Inhibition of viral protein expression: SDS-PAGE and immunoblotting
 - a. Lyse HFF cells in 1x NuPAGE sample buffer (Life Technologies) with protease inhibitors (cOmplete Protease Inhibitor Cocktail, Millipore Sigma).
 - b. Resolve samples in NuPAGE 4-12% Bis-Tris Gels (Life Technologies).
 - c. Transfer proteins to a nitrocellulose membrane (Bio-Rad).
 - d. Block the membrane in Odyssey Blocking Buffer (LI-COR) for 1 h.
 - e. Incubate the membrane with antibodies specific for individual proteins [anti-ICP4 (1:3,000), anti-ICP8 (1:5,000), anti-ICP27 (1:5,000), anti-UL30 (1:2,000), anti-GAPDH (1:10,000), and anti-HA (1:5,000)] for 1 h at room temperature or overnight at 4 °C.
 - f. Wash membrane 5 x 5 min with PBS-T (PBS with 0.1% Tween 20).
 - g. Incubate the membranes with secondary antibodies, IRDye 680RD (1:15,000, LI-COR) or IRDye 800CW (1:15,000, LI-COR), for 45 min.
 - h. Wash membrane 5 x 5 min with PBS-T (PBS with 0.1% Tween 20).
 - i. Detect near-infrared fluorescence using Odyssey (LI-COR) and quantify protein expression levels using Image J or ImageStudio V4 (LI-COR) (see Figure 5D).
2. Inhibition of lytic replication
 - a. Transduction of HFFs with SaCas9/sgRNA:
 - i. Prepare HFF cells at a density of 2×10^5 /well in a T25 flask one day prior to transduction
 - ii. Transduce with 2-5 ml of lentivirus (or at an MOI of 5) and 3-4 µg/ml of polybrene.
 - iii. The next day, replace the transduction medium with fresh medium and incubate cells for additional 2 days at 37 °C followed by puromycin treatment (1 µg/ml) for 7-10 days.
 - b. Lytic infection with wild-type HSV-1
 - i. Infect the transduced cells with wild-type HSV-1 at an MOI of 0.1 or 5 in phosphate-buffered saline (PBS) containing 0.1% glucose (wt/vol), 0.1% heat-inactivated BCS (v/v) for 1 h with shaking at 37 °C.
 - ii. Replace virus inoculum with DMEM containing 1% BCS and incubate at 37 °C.
 - iii. Harvest cells at various time points for viral replication by qPCR and titer.
 - iv. To determine viral titer, harvest media and cells and mix with the same volume of autoclaved powder milk and freeze down at -80 °C (addition of sterilized milk to regular media can stabilize HSV (Blaho et al., 2005)).
 - v. To isolate whole DNA (cellular and viral DNAs), isolate genomic DNA using the DNeasy Blood & Tissue kit according to the manufacturer's protocol.

c. Titration of viral yield:

- i. Thaw harvested cells in media/milk and repeat freeze-thaw cycle two times (3 cycles total).
- ii. Dilute the thawed samples in PBS containing 0.1% glucose (wt/vol), 0.1% BCS (v/v) (10 fold serially up to 6-8 logs) and incubate the serially diluted virus inoculum with the appropriate cell type monolayer, which is prepared one day prior, for 1 h with shaking at 37 °C.
- iii. Replace the virus inoculum with DMEM containing 1% BCS and 0.17% human IgG and incubate at 37 °C for 2-3 days until plaques appear.
- iv. Remove media and fix cells with 100% cold methanol for 10 min.
- v. Stain monolayer with GIEMSA stain and count plaques (see Figures 5B and 5C)

3. Inhibition of reactivation of quiescent virus (see Figure 6A)

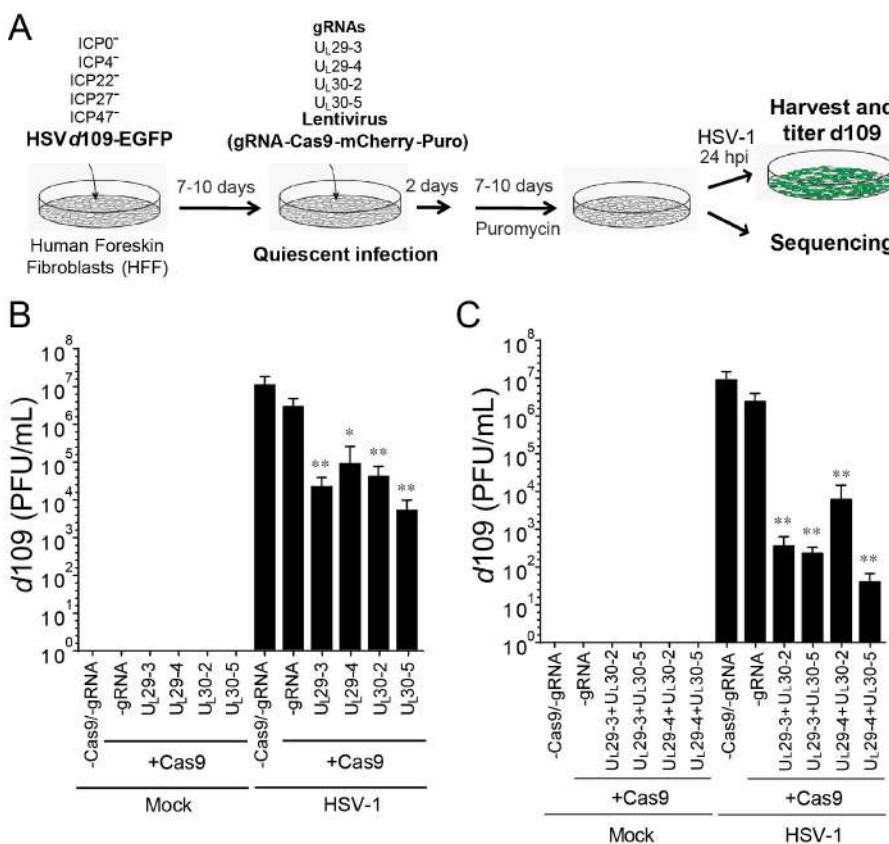


Figure 6. CRISPR/Cas9-induced mutagenesis of quiescent d109 genomes and effect on reactivation (Oh et al., 2019). A. Experimental scheme of SaCas9/sgRNA-mediated inhibition of reactivation of quiescent d109 genomes in HFFs. HFFs were infected with HSV-1 d109 virus to establish quiescent infection for 7-10 days and transduced with lentivirus expressing SaCas9 and sgRNAs for 7-10 days. B. and C. HFF were infected with d109 to establish quiescent infection for 7-10 days and transduced with lentivirus expressing SaCas9 and sgRNAs for 7-10 days. To reactivate quiescent d109 genomes, HFFs were superinfected with WT HSV-1 at an

MOI of 5 and harvested at 24 hpi. GFP-positive viral yields were determined by plaque assays on FO6 and V27 cells. The histogram shows the mean values and standard deviations of biological replicates (B and C: N = 5 and N = 7 respectively). All the sgRNA added conditions showed statistical significance compared to +Cas9/-gRNA (Ratio paired t test, *P < 0.05 and **P < 0.01).

- a. Establish quiescent *HSV-1* infection in HFF cells (Ferenczy and DeLuca, 2011):
 - i. Infect HFF cells with the *HSV-1 d109* mutant virus at an MOI of 10 in PBS containing 0.1% glucose (wt/vol) and 0.1% BCS (v/v) for 1 h with shaking at 37 °C.
 - ii. Incubate at 37 °C for 7-10 days in DMEM supplemented with 10% (v/v) FBS.
- b. Transduction of HFFs containing quiescent *HSV-1* viral genomes with SaCas9/sgRNA
 - i. Transduce the *d109* virus-infected cells with 2 or 10 ml of lentivirus (or at a MOI of 5) and 3-4 µg/ml of polybrene in 6-well plates or in T150 flasks.
 - ii. The next day, replace the transduction medium with fresh medium and incubate cells for 2 days at 37 °C followed by puromycin treatment (1 µg/ml) for 7-10 days.
 - iii. To reactivate quiescent *d109* virus, superinfect HFF cells with wild-type *HSV-1* at an MOI of 5 in PBS containing 0.1% glucose (wt/vol) and 0.1% BCS (v/v) for 1 h with shaking at 37 °C.
 - iv. Replace virus inoculum to DMEM containing 1% BCS for 24 h.
 - v. Harvest cells at 24 hpi and follow the protocol for titration of viral yield in Step C2c above using FO6 and V27 cells.
 - vi. To count the number of green plaques, fix the cells with 1% formaldehyde in PBS for 10 min.
 - vii. To calculate reactivated *d109*, count green plaques and subtract the number of GFP-positive plaques on V27 cells from the number of GFP-positive plaques on FO6 cells.
 - viii. Reactivation analysis: For the reactivation assay, it is important to include a control for recombination between WT *HSV-1* and *d109* which could transfer the GFP sequence to WT *HSV-1* and result in production of false GFP-positive plaques. We quantify *d109* viral reactivation using plaque assays by counting GFP-positive plaques on complementing FO6 cells. FO6 is a Vero-derived cell line expressing ICP4, ICP27, and ICP0 upon *HSV-1* infection, thereby complementing replication of *d109* virus (Samaniego et al., 1998). To measure these recombinant viruses, we count GFP-positive plaques formed on V27 cells, which express ICP27 (but not ICP4 and ICP0) upon infection with *HSV*. Because *ICP27* is replaced with *GFP* in *HSV-1 d109*, any recombinant GFP-positive but ICP27-negative *HSV* mutants that arise (but not *HSV-1 d109*), can replicate in V27 cells. To calculate the number of plaques originating from reactivated *d109* genomes, we subtract the number of GFP-positive plaques on V27 cells from the number of GFP-positive plaques on FO6 cells (see Figures 6B and 6C).

Data analysis

Controls: We include a control without sgRNA (SaCas9 only) for all experiments.

Statistical analysis: We use a minimum of three biological replicates (independent experiments that are performed using the same test at different times) to determine statistical significance. We use Prism 6 (Version 6.01) software (GraphPad Software) for statistical analysis. To determine statistical significance, we use Student's *t*-test, ratio paired *t*-test, or one-way ANOVA with Dunnett's multiple comparison test with two-sided (95% confidence level) (Oh *et al.*, 2019).

Recipes

1. Assay buffer (concentrations given are final concentration)

200 mM KCl

10 mM MgCl₂

20 mM Tris pH 8

Store at RT

2. Reaction Stop Buffer (Enzyme Assay)

30% glycerol by weight 15 g

0.5 M EDTA (6 ml)

2% SDS by weight 1 g

Small amount Bromophenol blue

Bring volume to 50 ml with water and vortex to mix thoroughly

Store at RT

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Competing interests

The authors declare no conflicts of interest or competing interests.

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An *in vitro* DNA Sensor-based Assay to Measure Receptor-specific Adhesion Forces of Eukaryotic Cells and Pathogens

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[Abstract] Motility of eukaryotic cells or pathogens within tissues is mediated by the turnover of specific interactions with other cells or with the extracellular matrix. Biophysical characterization of these ligand-receptor adhesions helps to unravel the molecular mechanisms driving migration. Traction force microscopy or optical tweezers are typically used to measure the cellular forces exerted by cells on a substrate. However, the spatial resolution of traction force microscopy is limited to ~2 μm and performing experiments with optical traps is very time-consuming.

Here we present the production of biomimetic surfaces that enable specific cell adhesion via synthetic ligands and at the same time monitor the transmitted forces by using molecular tension sensors. The ligands were coupled to double-stranded DNA probes with defined force thresholds for DNA unzipping. Receptor-mediated forces in the pN range are thereby semi-quantitatively converted into fluorescence signals, which can be detected by standard fluorescence microscopy at the resolution limit (~0.2 μm).

The modular design of the assay allows to vary the presented ligands and the mechanical strength of the DNA probes, which provides a number of possibilities to probe the adhesion of different eukaryotic cell types and pathogens and is exemplified here with osteosarcoma cells and *Plasmodium berghei* Sporozoites.

Keywords: Molecular force sensor, DNA-hairpin, Biomimetic surface, Receptor mediated forces, Molecular tension fluorescence microscopy, Plasmodium, Sporozoite

[Background] Motile cells and pathogens interact in many different ways with their environment (Parsons et al., 2010; Nan, 2017; Muthinja et al., 2018). For example, transmembrane receptors anchor single cells in their environment and allow them to interact with other cells (Hynes, 1992). Integrins, which are the main class of receptors connecting cells to the extracellular matrix, transmit forces in a bidirectional manner (Schoen et al., 2013). On the one hand, extracellular mechanical signals are transduced to the cytosol, where they trigger signaling cascades and thereby control various cellular functions like proliferation, differentiation and migration (Wang et al., 1993; Janmey and Miller, 2011; Totaro et al., 2017). On the other hand, forces generated by the cytoskeleton can be applied to the

extracellular environment, causing tissue shape changes and modulate cellular motility in specialized cells (Iskratsch et al., 2014).

To test for integrin-specific adhesion of cells, here, we first describe a method “option I” to prepare substrates presenting cyclic Arginin-Glycin-Aspartic acid (cRGD) ligands while non-specific surface interactions being prevented by a polyethylene glycol (PEG)-based passivation layer (Figure 1A). Of note, this method can be easily adopted to other receptors by varying the ligand.

To characterize the different aspects of cellular force transmission like absolute tension values, direction or the spatiotemporal distribution of forces several methods exist, which are based on the deformation of hydrogels, micropillars or unfolding of molecules, which differ in their spatial resolution and force range (Polacheck and Chen, 2016). One of the latest approaches visualizes traction forces exerted on single ligands via fluorescent DNA-based tension sensors (Wang and Ha, 2013; Blakely et al., 2014; Zhang et al., 2014). These double-stranded (ds) DNA or hairpin (particularly self-annealing single stranded (ss) DNA structures) sensors are immobilized on an in vitro surface and carry a ligand (e.g., cRGD) that enables for receptor (integrin)-specific cell interactions. Upon force, the dsDNA regions can be unfolded, which is detected with a fluorescent sensor (Figure 1B). In combination with standard wide-field or total internal reflection microscopy the spatiotemporal distribution of forces can be determined at high resolution, which is only limited by the optical resolution (~0.2 μm). The mechanical stability of dsDNA depends on its length and the GC content (Zhang et al., 2015). The force $F_{1/2}$ that is required to unfold 50% of the DNA sensors, has been calibrated in previous single-molecule studies between ~4-60 pN for varying DNA sequences (Wang and Ha, 2013; Zhang et al., 2014).

The herein presented method “option II” builds on DNA hairpins, which allow to quantify single-molecular, force transmission in the range of 4-19 pN (Zhang et al., 2014; Liu et al., 2016). We modified the DNA strands to enable coupling of various ligands in a modular manner depending on the receptors of interest. In the closed configuration, the fluorescent tag is in close proximity to a quencher and thus dark. Above a specific threshold force the DNA-hairpins unzip and elongate, which lowers the quenching efficiency and gives rise to a digital ON/OFF signal (Figure 1B) (Woodside et al., 2006). They can refold if the force no longer persists (Bonnet et al., 1998; Woodside et al., 2006), which is accompanied by a decrease in fluorescence. This is especially important for the characterization of cells with high turnover of adhesion formations like motile pathogens. Here, DNA hairpin sensors were immobilized via gold nanoparticles, which serve as additional fluorescence quenchers. This reduces the background signals induced by thermodynamic fluctuations as well as the photobleaching in comparison to other sensor surfaces (Lang et al., 2004; Liu et al., 2016). If the range of forces a certain receptor can transmit is not previously known, it is advisable to start with the lowest force probe as it quantitatively detects forces above its threshold. Next, the assay can be expanded by multiplexing sensors with different force thresholds and distinct fluorescent dyes to obtain semi-quantitative information on the distribution of cellular forces (Zhang et al., 2014).

Finally, we show how to combine substrate specific adhesion as established in “option I” with the molecular tension sensors “option II” allowing for traction measurements while assuring proper cell adhesion to the surface. This method “option III” can prevent frequent detaching of small and motile

cells like Plasmodium sporozoites, even if they show low binding affinities for the ligands coupled to the force sensors.

By immobilizing the molecular tension sensors on such adaptable substrates, they can be used in a wide range of biophysical studies of cell adhesion to extracellular ligands. We recently employed molecular tension sensors to study the specific adhesion between pathogenic agents like viral particles and mammalian cells (Wiegand et al., 2020). Here we provide a protocol to study the interaction of different cell types, including Plasmodium sporozoites, with specific matrix ligands. The rapid movement of the Malaria parasites is a crucial part in the infection cycle (Frischknecht and Matuschewski, 2017) and has been investigated with traction force microscopy (Münter et al., 2009) and laser tweezers (Quadt et al., 2016). Note that the molecular sensors semi-quantitatively detect distribution of forces transduced via single receptors, while they do not report on the overall forces transmitted per cell (Goktas and Blank, 2017) nor the direction of force without integration of further fluorescence polarization techniques (Brockman et al., 2018). Since the methods are very sensitive to changes in the experimental procedure, we recommend to carefully control each step of the protocol.

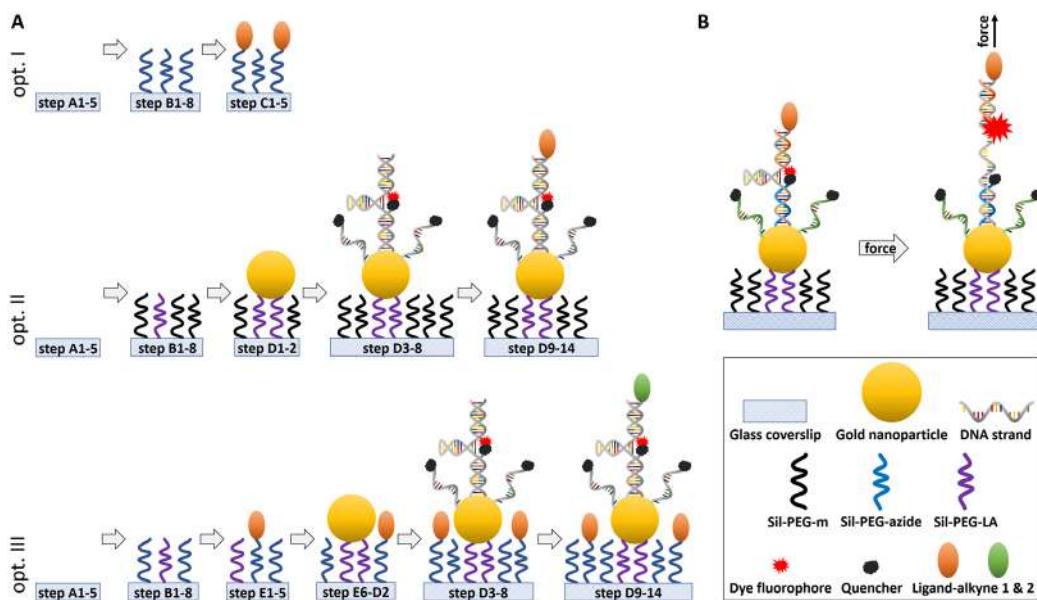


Figure 1. Schematic of the experimental design and working principle of the *in vitro* DNA-sensor force assay. A. Step-by-step representation of the individual steps in this protocol to generate ligand-presenting surfaces (Opt. I), surfaces with DNA-based force sensors (Opt. II) and combination of cell adhesion ligands presented in the background of the surface and DNA-based force sensor bearing another type of ligand (Opt. III). Briefly, glass coverslips are cleaned, passivated with a PEG-layer and cellular ligands, gold nanoparticles and DNA strands are immobilized on top. For details, the indicated steps lead to the corresponding sections in the procedure chapter. B. Design of the DNA hairpin force sensors in relaxed conformation and under load. ‘Hairpin’ ssDNA (grey) forms the backbone of the sensors and is linked to the Gold nanoparticle at the bottom and a ligand at the top. Top ssDNA (orange) is linked to the fluorescent dye, bottom ssDNA (blue) is linked to a quencher. Additional bottom ssDNA (green) increases the signal.

quenching of background fluorescence. Annealing procedure of the DNA hairpin probe is described in Recipe 8. When a force is applied the partially self-annealing ‘hairpin’ strand unfolds by rupturing the hydrogen bonds between nucleotides, lowering the quenching efficiency between the two dyes, and thus local increase in the fluorescent signal. Figure is not drawn to scale.

Materials and Reagents

Standard

1. 1.5 ml plastic reaction tube (e.g., Eppendorf, catalog number: 0030125150)
2. Aluminum foil
3. Cell hood
4. Erlenmeyer flask of different sizes
5. Flasks for buffers
6. Glass beaker of different sizes
7. Heat-resistant glass tub for oil bath
8. Pipettes of different sizes with tips
9. Schlenk flasks of different sizes
10. Fine-tipped metallic tweezers, e.g., #4, #7 (Sigma, catalog number: T6165)
11. Ice
12. Ultra-pure deionized water max. resistance of $18.2\text{ M}\Omega\cdot\text{cm}$, e.g., MilliQ purified
13. Inert gas (Ar)
14. Nitrogen gas
15. Schlenk line (Schlenk flask with wide opening, lid for Schlenk flask, connection tubing for Vacuum and Nitrogen gas)
16. Deionised (DI) water

Consumables

1. 20 x 20 mm coverslips #1 (Carl Roth, catalog number: H873.2)
2. DWK Life Sciences DURAN™ Filter Funnels (No. 3) (Fisher Scientific, catalog number: 09-841-090)
3. GE Healthcare illustra™, NAP-5 column (Fisher Scientific, catalog number: 10054394)
4. Glass coverslip holder (home-built)
5. Kimwipe tissues (Kimberly-Clark Worldwide, catalog number: 05517)
6. Molecular sieve 3 Å (Carl Roth, catalog number: 8487)
7. Pasteur pipettes ISO 7712 (230 mm) (Karl Hecht, catalog number: 40567002)
8. Petri dish, PS, 35/10 mm with vents (Greiner Bio-One, catalog number: 627102)
9. Rotilabo-syringe filter, CME, sterile (Carl Roth, catalog number: P818.1)
10. Silicon chamber (Ibidi GmbH, catalog number: 80841)

11. Teflon coverslip holder (home-built, commercially available, e.g., at Thermo Fisher Scientific, catalog number: C14784)
12. Silicon glue, Twinsil 22, Addition-curing duplicating silicone (Picodent GmbH, catalog number: 13001000)
13. Silicon oil for oil bath (Sigma-Aldrich, catalog number: 85409)

Chemicals

1. Gold Nanospheres, 9 nm (nanoComposix, custom size, 9 ± 2 nm, 0.05 mg ml^{-1} in tannic acid)
2. (+)-Sodium L-ascorbate (Sigma-Aldrich, catalog number: A7631)
3. 3-(triethoxysilyl)propylisocyanate (Sigma-Aldrich, catalog number: 413364)
4. Acetic acid ethyl ester (Carl Roth, catalog number: 6784)
5. Acetonitrile (CHROMASOLV for HPLC) (Honeywell International, catalog number: 60-002-31)
6. Aminoguanidine hydrochloride (Sigma-Aldrich, catalog number: A8835)
7. α -Silane- ω -alkyne PEG, $(\text{CH}_3\text{O})_3\text{-Si-PEG(3000)-C}\equiv\text{CH}$ (Rapp Polymere, catalog number: 133000-70-71)
8. α -Silane- ω -azido PEG, $(\text{CH}_3\text{O})_3\text{-Si-PEG(3000)-N}_3$ (Rapp Polymere, catalog number: 133000-5-71)
9. Copper sulfate (Sigma-Aldrich, catalog number: C1297)
10. Diethyl ether (Sigma-Aldrich, catalog number: 309966)
11. Dimethyl sulfoxide (for spectroscopy) (DMSO) (Merck, catalog number: 102950)
12. cRGD-alkyne (Biotrend Chemicals, custom synthesis: Cyclo(Arg-Gly-Asp-D-Phe-Pra))
13. Hydrogen peroxide 30% (Merck, catalog number: 107209)
14. LA-PEG(3400)-NH₂ (Biochempeg Scientific, catalog number: HE039005-3.4K)
15. Methanol (Carl Roth, catalog number: 0082)
16. N,N-Dimethylformamide (anhydrous, 99.8%) (DMF) (Sigma-Aldrich, catalog number: 227056)
17. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
18. Potassium hydroxide (for cleaning bath) (Sigma-Aldrich, catalog number: P5958)
19. Potassium phosphate monobasic (KH₂PO₄) (Sigma-Aldrich, catalog number: P5655)
20. QSY21 (Thermo Fisher Scientific, Invitrogen™, catalog number: Q20131)
21. Silane-PEG-methoxy, Sil-PEG(2000)-m (Biochempeg Scientific, catalog number: MF001020-2K)
22. Sodium carbonate (Na₂CO₃) (Carl Roth, catalog number: A135)
23. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
24. Sodium phosphate dibasic (Na₂HPO₄) (Sigma-Aldrich, catalog number: 255793)
25. StarRed-NHS (Abberior) (Abberior, catalog number: 1-0101-011-3)
26. Sulfuric acid 96% (VWR CHEMICALS, catalog number: 1.08131.1000)
27. Toluene (Merck, catalog number: 108325)
28. Triethylamine (Sigma-Aldrich, catalog number: 471283)
29. Triethylammonium acetate (TEAA) (Sigma-Aldrich, catalog number: 69372)

30. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (Sigma-Aldrich, catalog number: 762342)
31. Click reaction mixture (CuAAC) (see Recipes)
32. Dried toluene (see Recipes)
33. Sil-PEG(3500)-LA (see Recipes)

DNA strands (custom synthesis by Integrated DNA Technologies, Inc.)

Note: Self-annealing sections underlined, top strand annealing dotted, bottom strand annealing dashed; a list of the hairpins used in literature and their calibration can be found in Zhang et al., 2014 and Liu et al., 2016.

1. Top: 5'- /5AmMC6/CGC.ATC.TGT.GCG.GTA.TTT.CAC -3'
2. Bottom NH₂-quencher:
5'- /5ThioMC6-D/TTT_GCT_GGG_CTA_CGT_GGC_GCT_CTT/3AmMO/ -3'
3. Hairpin 4.2 pN:
5'- /5AzideN/GTG.AAA.TAC.CGC.ACA.GAT.GCG.TTT - GTA TAA ATG TTT TTT TCA TTT ATA
C - TTT AAG AGC GCC ACG TAG CCC AGC AAA/3ThioMC3-D/ -3'
4. Hairpin 12 pN:
5'- /5AzideN/GTG.AAA.TAC.CGC.ACA.GAT.GCG.TTT - GGG TTA ACA TCT AGA TTC TAT TTT
TAG AAT CTA GAT GTT AAC CC - TTT AAG AGC GCC ACG TAG CCC AGC AAA/3ThioMC3-D/ -3'
5. Hairpin 19 pN:
5'- /5AzideN/GTG.AAA.TAC.CGC.ACA.GAT.GCG.TTT - CGC CGC GGG CCG GCG CGC GGT
TTT CCG CGC GCC GGC CCG CGG CG - TTT AAG AGC GCC ACG TAG CCC AGC AAA/3ThioMC3-D/ -3'
6. Labeled DNA strands (see Recipes)
7. Annealed DNA strands (see Recipes)

Enzymes

1. StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific, Gibco®, catalog number: A1110501)

Medium

1. Albumin Fraction V (Carl Roth, catalog number: 8076)
2. Fetal Bovine Serum (USA) (Sigma-Aldrich, catalog number: F2442)
3. MEM α (Thermo Fisher Scientific, Gibco®, catalog number: 12561)
4. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco®, catalog number: 15140148)
5. RPMI 1640 Medium (Thermo Fisher Scientific, Gibco®, catalog number: 11-875)
6. Sodium Pyruvate 100 mM (100x) (Thermo Fisher Scientific, Gibco®, catalog number: 11360070)
7. Microscopy U2OS medium (see Recipes)

8. Microscopy sporozoite gliding medium (see Recipes)
9. PBS buffer (see Recipes)

Cell lines

1. *Plasmodium berghei* ANKA strain (BEI resources (ATCC), catalog number: MRA-871)
2. *Plasmodium berghei* NK65 strain, CS-GFP line (optional usage, see Step H4) (not commercially available, requestable from cooperating parasitological institutes; further information can be found in Natarajan et al., 2001)
3. U2OS, osteosarcoma cells (ATCC, catalog number: HTB-96)

Equipment

1. 300 °C Oven
2. Freezer (-20 °C and -80 °C)
3. Fridge (4 °C)
4. Fume hood
5. Centrifuge (Thermo Fisher Scientific, model: Heraeus Biofuge Pico)
6. Centrifuge (Thermo Fisher Scientific, model: Heraeus Multifuge 1S-R; catalog number: 75004331)
7. Hot Plates Accessories (IKA, model: ETS-D5, catalog number: 0003378000)
8. HPLC (GE Comp Healthcare Life Sciences, model: ÄKTA Pure Chromatography System)
9. HPLC column (Phenomenex, model: Luna 5u C18(2)-RP-HPLC, catalog number: 00G-4252-N0)
10. Lyophilisator (Labconco, model: FreeZone Plus 2.5 Liter Cascade Benchtop Freeze Dry System, catalog number: 7670020)
11. Magnetic Stirrers (IKA, model: C-MAG HS 7, catalog number: 0003581200)
12. Wide-field, inverted fluorescence microscope (Applied Precision, model: DeltaVision™ Elite Microscope System, based on an Olympus IX71 body) with home-built environmental chamber (commercially available incubators e.g., at OKOLAB)
13. Microscope filter set (Applied Precision, model: DeltaVision™ Elite Filter Sets)
14. Microscope objective (Olympus, model: Objective 60x/1.4 NA PlanApo IX70)
15. Sonicator (EMAG Technologies, model: Emmi-H22, catalog number: 98217-DE)
16. Spectrophotometer (NanoDrop Technologies, model: PEQLAB Nano Drop ND-1000)
17. Thermal cycler (Bio-Rad Laboratories, model: Bio-Rad T100, catalog number: 1861096)

Software

1. Fiji is just ImageJ (Version 1.52e; <https://fiji.sc/>) (Schindelin et al., 2012)
2. Stack contrast adjustment, plugin for Fiji (Michálek et al., 2007)

Procedure

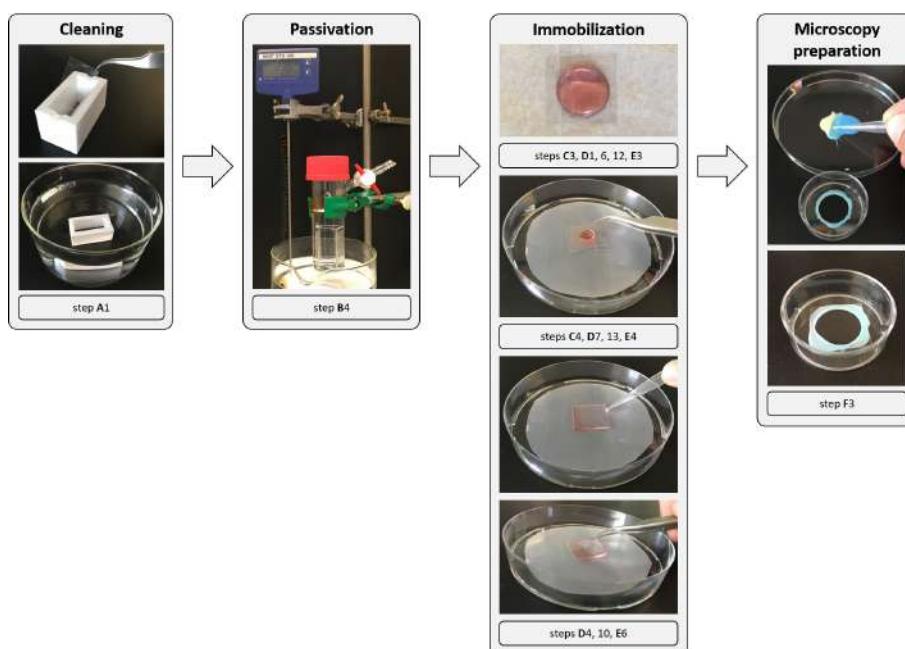


Figure 2. Overview of the experimental procedure. Glass coverslips are handled with a pair of tweezers and inserted in a Teflon holder (white), which is transferred into a beaker with Piranha solution for cleaning (Procedure A). For passivation of the coverslips they are transferred into a glass holder and incubated with the Sil-PEG passivation solution in a Schlenk flask at 80 °C in an oil bath. The Schlenk flask can be attached to a vacuum and nitrogen line via its valve to provide an inert reaction container (not shown, Procedure B). After cleaning and drying the coverslips, specific ligands and gold nanoparticles (red solution) are immobilized by incubating a drop of the respective solution in-between two coverslips. To remove the coverslips again from each other, additional water or PBS is injected with a pipette and the upper coverslip is lifted with a pair of tweezers (Procedures C-E). To observe the surfaces at an inverted microscope they are glued with a two-component silicon glue (yellow and blue gel) into a Petri dish with a custom-made hole in the center (2 mm smaller than the coverslip) (Procedure F). The final device is shown in the last figure, where medium and cells can be added before transferring to a microscope. Further information is provided at the steps indicated below the pictures.

A. Cleaning of glass surfaces

1. Clean and activate coverslips in a Teflon holder in ~100 ml freshly prepared ‘Piranha solution’ [3:1 (v/v) sulfuric acid: hydrogen peroxide] for 1 h. Make sure the coverslips are completely covered by solution (Figure 2, cleaning). Stir the Piranha solution e.g., with an acid-resistant Pasteur pipette.

CAUTION: Piranha solution is very corrosive and reacts exothermic. It must not get in contact with organic solvents. Perform every work step containing Piranha solution under a fume hood with constant ventilation.

Note: From here on transfer and handle the coverslips only with tweezers (Figure 2).

2. Remove ‘Piranha solution’ from the coverslips by carefully decanting it into an appropriate waste container according to the safety regulations of your institute, e.g., discarding after 10-20x diluted in water.
3. Rinse coverslips 3 times with DI water and twice with ultra-pure water.
4. Sonicate coverslips in ultra-pure water once for 5 min in ultrasonic cleaner at ~60 watt (standard sonication power).
5. Dry surfaces in a nitrogen stream by holding them carefully with tweezers.

Check point: Observe by eye that no stripes or drops of water remain else repeat Steps A3-A4 or discard the coverslip.

B. Passivation of glass surfaces

1. Transfer dried coverslips to a glass holder with tweezers and avoid touching them in the center.
2. Prepare passivation solution as indicated in Table 1 (depending on the desired surface Opt. I-III, see Figure 1A) under continuous protective nitrogen atmosphere in a Schlenk flask (Figure 2, passivation). All steps involving organic solvents (Steps B2-B7) shall be performed following the safety regulations of your institute under a fume hood with constant ventilation. Create a protective atmosphere by connecting the Schlenk flask to a vacuum line and nitrogen supply. First vacuum-empty the flask, then flush with nitrogen and reduce the nitrogen stream in the following to add the powdery PEG, dried toluene and triethylamine.

Table 1. Passivation compositions for the three different type of surfaces (Option I-III).

Option I should be chosen when the adhesion of cells to a specific ligand is to be tested, Option II to access the forces at such specific ligands and Option III when ligand-specific adhesion to the surface should be granted independent of the force measurements of specific ligands.

Reagent	Opt. I – ligand surface (see Procedure C) [abs. (mol. ratio)]	Opt. II – hairpin force sensors (see Procedure D) [abs. (mol. ratio)]	Opt. III – hairpin force sensors on ligand surface (see Procedure E) [abs. (mol. ratio)]
Sil-PEG(2000)-m		6 mg (95%)	
Sil-PEG(3000)-azide	6 mg (100%)		6 mg (95%)
Sil-PEG(3000)-alkyne	or 6 mg (100%)		or 6 mg (95%)
Sil-PEG(3500)-LA		0.35 mg (5%)	0.35 mg (5%)
Dried toluene	20 ml	20 ml	20 ml
Triethylamine	a drop (~100 µl)	a drop (~100 µl)	a drop (~100 µl)

3. Add coverslips under protective atmosphere into the Schlenk flask in the solution and close the lid and gas supply. The reaction is now kept under protective atmosphere.

4. Heat tube overnight at 78-80 °C in an oil bath. The oil bath can be set up combining the heat-resistant glass tub filled with oil on the magnetic stirrer. The additional thermostat keeps the temperature constant.
5. Wash passivated coverslips by sonication in ethyl acetate for 5 min within their glass holder.
6. Wash passivated coverslips by sonication in methanol for another 5 min within their glass holder.
Pause point: It is possible to store passivated glass surfaces in methanol for up to 5 days if they are not used immediately.
7. Dry surfaces in a nitrogen stream by holding them carefully with tweezers. Nitrogen stream can be dosed carefully with a compressed air gun. Make sure not to scratch the passivated surfaces during the entire procedure.
Check point: As control for proper passivation add a dye (e.g., 100 nM StarRed in PBS) or seed adhesive cells on a passivated versus a non-passivated coverslip and observe their attachment to the surface under a microscope.
8. For reuse, clean the used Schlenk flask and glass sample holder from PEG residue in a potassium hydroxide bath overnight and wash them afterwards with DI water.

C. Option I—Immobilization of specific cellular ligands

1. Prepare 100 µl of the reaction mixture for copper-catalyzed azide-alkyne cycloaddition (CuAAC, see Recipe 7) containing the desired cellular ligand.
Note: Choose appropriate alkyne or azide functionalized PEG for azide or alkyne-ligands, respectively.
2. Prepare a humidity chamber with parafilm on the bottom and a wet tissue inside a Petri dish where coverslips are placed onto (Figure 2, immobilization). Parafilm is used to hold the coverslips in their spot and to ease the separation of ‘sandwiched’ glasses later on.
3. Place a drop of 100 µl click mixture on top of a passivated and dried coverslip.
4. Place a second, identical coverslip on top of the first one.
5. Incubate the ‘sandwich’ for 2 h at room temperature (RT).

D. Option II—Immobilization of DNA hairpin force sensors

1. Place a drop of 250-300 µl of the gold nanoparticle solution on top of a passivated and dried coverslip.
2. Incubate the coverslips for 30 min at RT.
3. In the meantime, hybridize hairpin force sensors using ‘top’ and ‘bottom’ and ‘hairpin’ ssDNA. Use DNA mixture and annealing program as indicated (see Recipe 8). If DNA strands are purchased unlabeled, DNA and fluorophore should be coupled in a previous step (see Recipe 4 for labeling of DNA strands below).
4. Separate the surfaces by carefully pipetting 300 µl ultra-pure water in-between the glasses and pick them up with a pair of tweezers (Figure 2, Immobilization). Clean them from supernatant gold nanoparticle solution by thoroughly rinsing with ~10 ml ultra-pure water.

Check point: Confirm successful immobilization of gold nanoparticles by the pink shine of the coverslip.

5. Remove excess liquid with tissues over the coverslip edges without letting them run completely dry or touching them.
6. Place a drop of 100 µl DNA mixture on top of a gold-coated coverslip.
7. Place a second, identical coverslip with the gold nanoparticle-coated side facing down on top of the first one.
8. Incubate the ‘sandwich’ overnight at 4 °C under exclusion of light.
9. Prepare 100 µl of the reaction mixture for CuAAC (see Recipe 7) containing tripeptide/ligand.
10. Separate and clean the surfaces carefully from DNA mixture by washing in PBS (analogous to Step D4).
11. Remove excess liquid with tissues over the coverslip edges without letting them run completely dry or touching them.
12. Place a drop of 100 µl click mixture on top of a DNA-coated coverslip.
13. Place a second, identical coverslip with the DNA-coated side facing down on top of the first one.
14. Incubate the ‘sandwich’ for 2 h at RT in the humidity chamber under exclusion of light to avoid bleaching of the fluorophores.

E. Option III—Combination of specific cellular ligand and DNA hairpin force sensors

1. Prepare 100 µl of the 1st reaction mixture for CuAAC (see Recipe 7) containing 1st ligand.
2. Prepare a humidity chamber with a wet tissue inside a Petri dish and flat parafilm on the bottom where coverslips are placed onto.
3. Place a drop of 100 µl click mixture on top of a passivated and dried coverslip.
4. Place a second, identical coverslip on top of the first one.
5. Incubate the ‘sandwich’ for 2 h at RT.
6. Separate and clean the surfaces carefully from click mixture by washing in PBS (analogous to Step D4).
7. Further steps are equal to ‘D) Immobilization of DNA hairpin force sensor immobilization’ Steps D1-D14 using the 2nd ligand at Step D9.

F. Sample preparation for microscopy

1. Separate and clean the surfaces carefully from click mixture by washing in PBS (analogous to Step D4).
2. Remove excess liquid with tissues over the coverslip edges without letting them run completely dry or touching them.
3. Glue coverslips with the functionalized surface facing up into Petri dishes with custom made holes (2 mm smaller than the coverslips) in the bottom. Therefore, mix ~200 µl of Twinsil 22 silicon 1:1 e.g., with a pipette tip and apply the glue on the edges of the hole, such as the coverslip completely covers the hole and is surrounded by glue.

4. Wait curing time of the glue (~ 5 min, check with the remaining glue). If necessary, clean surface by washing in PBS to remove glue contamination.

Check for successful immobilization of cellular ligands and force sensors by comparing cellular attachment (Procedure G) and homogenous background fluorescence, respectively, of only passivated coverslips versus those with immobilized ligands (Procedure C) or force sensors (Procedure D).

G. Assays with cells (U2OS)

1. Bring chamber for cell observation to 37 °C and 5% CO₂ atmosphere.
2. Fill 2-3 ml cellular medium (0.5% FBS) (see Recipe 5) in the Petri dishes prepared in step F covering the functionalized coverslip. Use medium with low FBS content to prevent unspecific ligand interactions and improve microscopy. Use a cell culture fume hood to prevent medium or cell culture contamination.
3. Harvest adherent cells from culture flask by washing with PBS and detach with Accutase following standard protocols (e.g., Basic Cell Culture Protocols (Helgason and Miller, 2004)).
Note: Accutase gently detaches cells for faster spreading results.
4. Seed cells in diluted concentration to allow an undisturbed spread. In case of U2OS 10⁵ cells reach an adequate density (calculated from T-25 and 35 mm dish flask surface for around 1:10 dilution).
5. Wait until cells settle down and start productively spreading on the surfaces (10-30 min).

Note: Force transmission is observed best 30 min post seeding cells and appears as bright dots and radial stripes of opened DNA hairpin sensors (Figure 3).

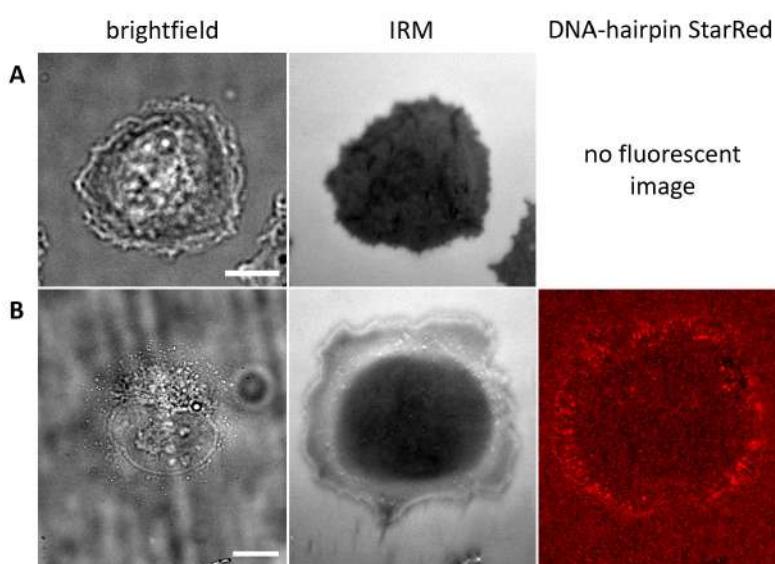


Figure 3. U2OS cells adhering and exerting forces on specific ligand presenting surfaces.

Representative bright-field, interference reflection microscopy (IRM) and fluorescence wide-field images of U2OS cells 15-20 min after seeding on the surfaces with (A) option I: cRGD-functionalization and (B) option II: cRGD-linked DNA hairpin sensors (4.2 pN opening force;

StarRed-fluorophore). Scale bars = 10 μm .

H. Assays with sporozoites

1. Try to keep the edges of the coverslip as dry as possible, whereas the center of the functionalized surface should be kept humid. This eases the silicon chamber founding.
2. Press a silicon chamber with its sticky side on the edges of the functionalized coverslip to minimize the cellular distribution area (Note: silicon sticks best on dry glass). Purchased multi-well chambers can be cut to a fitting size. This step can be skipped, but eases to generate an adequate parasite density.
3. Fill 15 μl sporozoite medium (6% BSA) (see Recipe 6) into the silicon chamber.
4. Add 15 μl ice-cooled PBS containing freshly isolated sporozoites from 6-8 pairs of well infected mosquitoes' salivary glands (for a detailed protocol of mosquito infection and sporozoite collection see Prinz et al., 2017) resulting in 3%-BSA-medium.

Notes:

- a. FBS-reduced cellular medium (0.5% FBS) can be used as well.
 - b. Do not keep salivary glands on ice for prolonged times as this could harm the sporozoite motility.
 - c. Usage of GFP-tagged sporozoites eases the selection of well infected salivary glands as well as their isolation. They can be employed on ligand-presenting surfaces (option I, see Figure 4A), but care must be taken to choose proper filter sets when using them together with force sensors (Figure 4B) to avoid fluorescent bleed through.
5. Spin cells down to surface in a centrifuge at 2,000 $\times g$ for 3 min. Well-founded silicon chamber can stand this procedure.
 6. Motility can be observed directly after attaching to the surfaces for around 30 min (Figure 4).

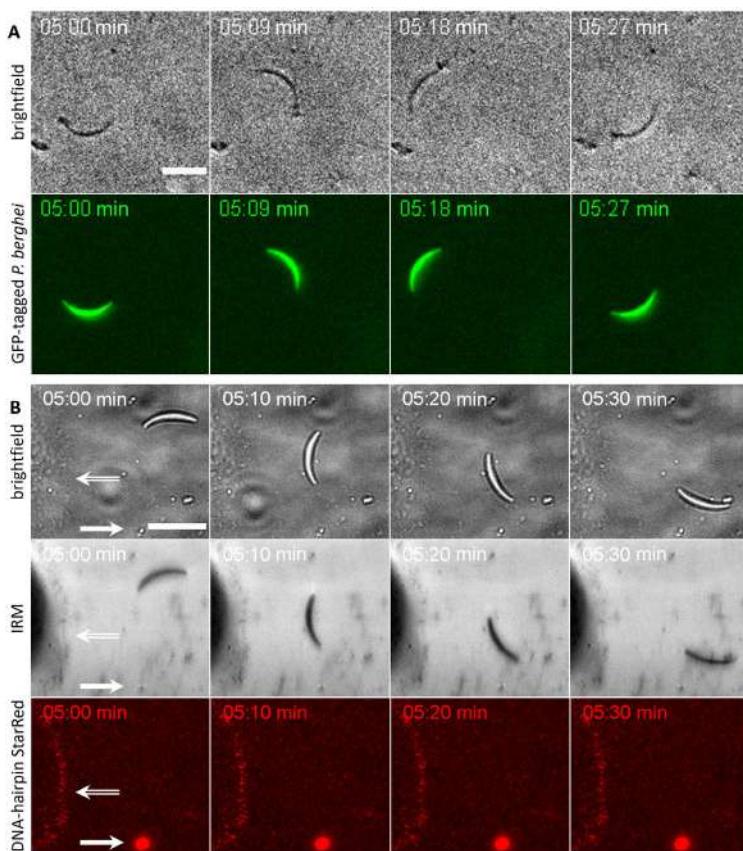


Figure 4. Sporozoites (*P. berghei*) gliding on specific ligand presenting surfaces.

Representative bright-field, interference reflection microscopy (IRM) and fluorescence wide-field time lapse images of (A) GFP-expressing sporozoites 5 min after seeding on the surfaces with option I: cRGD-functionalization and (B) WT sporozoites 5 min after seeding on the surfaces with option II: cRGD-linked DNA hairpin sensors (4.2 pN opening force; StarRed-fluorophore). For Option II Osteosarcoma cells (hollow arrow) were seeded together with the sporozoites as a positive control for the DNA-based force sensors (see Figure 3). Sporozoites adhere and glide on both surfaces, however, no tension signal was detected with the DNA hairpin sensors. This might be due to sub-threshold forces, few surface interactions or too short adhesion times (sporozoites move at ~2 μ m/s), which do not allow for detectable unfolding of the tension probes, while osteosarcoma cells adhering on the same substrates gave rise to fluorescent signals (hollow arrow). Bright fluorescent spots (full arrow) are auto fluorescent remnants from the purification of Sporozoites out of mosquitoes' salivary glands. Scale bars = 10 μ m.

I. Microscopy settings

1. Imaging was performed on a fluorescence wide-field microscope (Olympus) equipped with a custom-made environmental chamber for cellular conditions (37 °C; 5% CO₂). Sporozoites were usually imaged at RT without CO₂ supply but we also successfully performed gliding motility assays under cellular conditions.

2. Brightfield images were taken with a polarized light filter set.
3. Fluorescence microscopy images were taken with excitation bandpass filter 632/22 nm and emission filter 679/34 nm.
4. Interference reflection microscopy (IRM) images were taken with excitation bandpass filter 542/27 nm in IRM mode (set emission bandpass filter 525/48 nm) with minimal exposure time and intensity. IRM observation can be thus used as a low-bleaching control for cell adherence to the surface during the imaging process.

Data analysis

Receptor surfaces (option I), which do not contain force sensors, can be used for a qualitative testing of the cellular adherence properties to defined ligands on an otherwise non-adherent, PEG-ylated surface. Brightfield imaging or IRM allows to validate if cells adhere tightly to the surface, such as no fluorescence microscopy is required for this approach.

Furthermore, quantitative analysis of the settling and spreading time of cells on the surface (data analysis see Schaufler *et al.*, 2016) or of the gliding motility in case of motile sporozoites (data analysis see Prinz *et al.*, 2017) can be used for comparable data analysis between different cell/parasite lines or different ligands.

DNA force sensor surfaces (options II and III) provide a digital ON-OFF signal at a specific threshold force. Fluorescence microscopy images of the sensor surface provide spatial, temporal, and semi-quantitative information on the exerted forces, indicated by the transient appearance (hairpins) of the fluorescent signal. Therefore, fluorescence microscopy images were first adjusted for brightness and contrast for better visibility of the signals with the Fiji software. For time lapse imaging, ‘Stack contrast adjustment’ plugin is used to correct for the photobleaching. A semi-quantitative force readout can be generated from the local brightness reflecting the relative amount of dsDNA opening and displayed via a color-coded LUT e.g., “spectrum” (for more details see Zhang *et al.*, 2014, Supplementary Figure 7). However, this quantitative analysis is limited by the optical resolution, since typically multiple sensors will be contained per diffraction limited spot.

To narrow down the absolute traction values, the experiment can be repeated with DNA sensors of different rigidity by varying the DNA sequence. Multiple of these sensors coupled to different fluorophores can be combined on one surface for multiplex results (see Zhang *et al.*, 2014).

Notes

1. Prepare aliquots of appropriate volumes for frozen medium or solution to avoid repeated freeze thaw cycles and elongate their shelf lifetime.
2. Separate and clean the surfaces from reaction mixture by carefully adding 300 µl PBS inside the sandwich with a pipette. Subsequently lift off top coverslip and wash them in PBS. Make sure you never scratch the surfaces during the entire procedure.

3. Avoid drying out of the surfaces at any time since retracting liquids can produce artefacts caused by surface tension on surfaces.
4. Other fluorescent dyes and quenchers with NHS function can be used for the labeling of DNA depending on the availability of filters at the microscope. Consider dyes with a high quantum yield and an emission wavelength close to the particle resonance peak of gold nanoparticles for stronger quenching (Kang et al., 2011). Note, that cellular autofluorescence, especially from nucleus, can overlap sensor signals from underneath the cell. For smaller cells with a high nuclear to body ratio (e.g., *Plasmodium* sporozoites), far-red dyes like StarRed can minimize this signal-autofluorescence interference.

Recipes

1. PBS buffer
 - a. Prepare PBS as indicated:

NaCl	8.006 g (c _{final} : 137 mM)
KH ₂ PO ₄	1.375 g (c _{final} : 10.1 mM)
KCl	201.3 mg (c _{final} : 2.7 mM)
Na ₂ HPO ₄	255.5 mg (c _{final} : 1.8 mM)
 - b. Add 0.8 L ultra-pure water
 - c. Adjust pH to pH 7.4 by adding a few Milliliter HCl (1 M)
 - d. Add ultra-pure water to a total volume of 1 L
 - e. Store buffer at RT
2. Sil-PEG(3500)-LA
 - a. Solve 145 µmol NH₂-PEG(3400)-LA in 3 ml DMF in a small Schlenk flask under nitrogen atmosphere with a magnetic stir bar
 - b. Add 159.5 µmol 3-(trimethoxysilyl)propylisocyanate (1.1-times the molecular amount of PEG)
 - c. Stir overnight at room temperature
 - d. Cool solution down to 4 °C
 - e. Add cooled diethyl ether for 1 h
 - f. Wash the reactant with cooled diethyl ether over a glass frit (No. 3)
 - g. Dry gained Sil-PEG-LA from frit overnight under vacuum
 - h. Store the powder at -20 °C under inert gas
3. Dried toluene
 - a. Dry molecular sieve thoroughly in an oven
 - b. Vacuum an empty Erlenmeyer tube and add a nitrogen atmosphere
 - c. Add toluene under protection atmosphere and close flask properly
 - d. Wait several hours to get rid of excessive water in the toluene
 - e. Store at room temperature under nitrogen atmosphere

4. Labeling of DNA strands

- a. Dissolve DNA in ultra-pure water to a concentration of 1 mM
- b. Dissolve dye-NHS respectively quencher-NHS in dried DMSO to 10 mM
- c. Dissolve 105.99 mg Na₂CO₃ in 1 ml ultra-pure water
- d. Mix 60 µl solved DNA top strand (c_{final} : 0.2 mM), 60 µl solved dye-NHS (c_{final} : 2 mM) and 30 µl Na₂CO₃ solution (c_{final} : 0.1 mM) in 150 µl PBS. Proceed the same way with DNA bottom strand and the quencher-NHS
- e. Adjust pH to 8.5
- f. Stir mixture overnight at room temperature under the exclusion of light
- g. Purify mixture in NAP-5 column as indicated in the user manual of the manufacturer
- h. Purify mixture in HPLC (gradient high-pressure liquid chromatography). Use Luna 5u C18(2)-RP-HPLC column, mobile phases are triethylammonium acetate (TEAA) (0.1 M) and acetonitrile with a gradient from 10% to 50% in 90 min and from 50% to 90% in 20 min
- i. Collect fraction showing both maximum absorption peaks at the same time for DNA (260 nm) and dye
- j. Freeze product to -80 °C and lyophilize it after that to get rid of the solvents
- k. Resuspend pure product in ultra-pure water
- l. Determine the concentration with a nanodrop spectrophotometer measuring the absorbance $A_{DNA; 1mm}$ of DNA at 260 nm and the absorbance $A_{dye; 1mm}$ at the excitation wavelength of the respective dye/quencher
- m. Calculate concentration from three different measurements via:

$$c_{DNA}[M] = \frac{(A_{DNA; 1mm} - A_{dye; 1mm} \times CF_{260\text{nm}})}{\varepsilon_{DNA}} \times 10 \text{ mm/cm}$$

$$c_{dye}[M] = \frac{A_{dye; 1mm}}{\varepsilon_{dye}} \times 10 \text{ mm/cm}$$

Note: Take the extinction coefficient ε_{dye} and correction factor CF_{260nm} for the dye spectrum at 260 nm wavelength into account.

- n. Store resulting strand at 4 °C under the exclusion of light

5. Microscopy U2OS medium

- a. Prepare cell medium (0.5% FBS) as indicated:

MEM	48.75 ml
Pyruvate	0.5 ml
Penicillin/Streptavidin 100x	0.5 ml
FBS	0.25 ml

- b. Mix and filter medium under sterile conditions (hood)

- c. Store medium at 4 °C

6. Microscopy sporozoite gliding medium

- a. Prepare sporozoite medium (RPMI + 6% BSA) always freshly as indicated:

RPMI	44.5 ml
Penicillin-streptomycin	2.5 ml
Albumin fraction V	3 g

- b. Dissolve albumin thoroughly

- c. Spin down unsolvable particles at 13k rpm for 5 min in the Heraeus Biofuge

7. Click reaction mixture (CuAAC) (adapted from Hong et al., 2009)

- a. Prepare click reaction buffer as indicated:

NaCl	87.7 mg (c_{final} : 150 mM)
Na ₂ HPO ₄	114.7 mg (c_{final} : 80.8 mM)
KH ₂ PO ₄	25.9 mg (c_{final} : 19 mM)

Add 8 ml ultra-pure water

- b. Adjust the buffer to pH 7.4 by adding a few Microliter HCl (1 M)

- c. Add ultra-pure water to a total volume of 10 ml

- d. Dissolve aminoguanidine in ultra-pure water to a concentration of 100 mM

- e. Dissolve copper sulfate in ultra-pure water to a concentration of 20 mM

- f. Dissolve cRGD-alkyne in ultra-pure water to a concentration of 10 mM

Note: Other tripeptides and ligands containing an alkyne function can be used equivalently.

For azide-functionalized molecules the alkyne reaction partner is substituted and treated equally in this recipe.

- g. Dissolve sodium ascorbic acid in ultra-pure water to a concentration of 100 mM
- h. Dissolve THPTA in ultra-pure water to a concentration of 50 mM
- i. Premix 3 µl THPTA with 1.5 µl copper solutions thoroughly
- j. Mix 87.5 µl click reaction buffer with 1 µl cRGD-alkyne (c_{final} : 0.1 µM), 1.5 µl THPTA/copper premix (c_{final} : 0.5 µM/0.1 µM), 5 µl aminoguanidine (c_{final} : 5 µM) and 5 µl ascorbate (c_{final} : 5 µM)
- k. Store buffer at RT. Store aminoguanidine and copper solutions at 4 °C, peptide-alkyne and THPTA solutions at -20 °C. Prepare ascorbate solution always directly before use

8. DNA annealing

- a. Dissolve DNA strands in ultra-pure water to concentration of 10 µM. In case of labeled top DNA strand, use the calculated values form the spectrophotometer measurement after resuspending. Store DNA strands at 4 °C, store hairpin strand at -20 °C
- b. For hairpin sensors, mix 3 µl top DNA (c_{final} : 0.3 µM), 3 µl bottom DNA (c_{final} : 0.3 µM) and 3.3 µl hairpin DNA (c_{final} : 0.33 µM) to 70.3 µl NaCl buffer (1 M)
- c. Anneal the strands in thermal cycler. Program the thermal cycler: heat 10 min at 95 °C (denaturation), hold 4 s at 95 °C (annealing), repeat last step 399x while cooling down 0.2 °C at each step, final hold at 12 °C
- d. Add 20.4 µl additional bottom DNA (c_{final} : 2.04 µM) to the annealed DNA

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Competing interests

The authors declare that no competing financial interests exist.

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***Candida albicans* Culture, Cell Harvesting, and Total RNA Extraction**

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[Abstract] Transcriptional analysis has become a cornerstone of biological research, and with the advent of cheaper and more efficient sequencing technology over the last decade, there exists a need for high-yield and efficient RNA extraction techniques. Fungi such as the human pathogen *Candida albicans* present a unique obstacle to RNA purification in the form of the tough cell wall made up of many different components such as chitin that are resistant to many common mammalian or bacterial cell lysis methods. Typical *in vitro* *C. albicans* cell harvesting methods can be time consuming and expensive if many samples are being processed with multiple opportunities for product loss or sample variation. Harvesting cells via vacuum filtration rather than centrifugation cuts down on time before the cells are frozen and therefore the available time for the RNA expression profile to change. Vacuum filtration is preferred for *C. albicans* for two main reasons: cell lysis is faster on non-pelleted cells due to increased exposed surface area, and filamentous cells are difficult to pellet in the first place unlike yeast or bacterial cells. Using mechanical cell lysis, by way of zirconia/silica beads, cuts down on time for processing as well as overall cost compared to enzymatic treatments. Overall, this method is a fast, efficient, and high-yield way to extract total RNA from *in vitro* cultures of *C. albicans*.

Keywords: *Candida albicans*, RNA extraction, Cell harvest, Fungal transcription, RNA isolation

[Background] The need for fast, reproducible, and efficient RNA extraction techniques has grown significantly over the last several years due to the steady increase in use of RNA sequencing and other expression analysis techniques that have become more affordable and faster with improvements in sequencing technology. There are many different kits and protocols out there from various companies and labs that attempt to meet this need. However, methods that are built specifically for one type of fungi may not be usable for another, and kit platforms can often fall short by way of being too broad in their application. Here we describe a cell culture, harvest, and RNA extraction method for the pathogenic fungus, *Candida albicans*, that utilizes a combination of techniques to give both high yield and high-quality RNA in a consistent and efficient manner.

One of the main attributes unique to this approach is the harvest of cells via vacuum filtration rather than by centrifugation. Centrifugation of a 25 ml culture of filamentous cells, as is used in this protocol, must be done over a period of 5 min in order to pellet the cells enough to aspirate the growth media. This additional time before freezing the cells and halting cellular processes opens the door for unwanted transcriptional changes. Previous studies have shown that yeast cells can alter their expression profiles well within the 5 min that is needed to spin cells down (Dikicioglu *et al.*, 2011). It is then of critical importance to shorten the time between the incubation/growth period and when the cells are frozen, and

vacuum filtration serves this purpose well. It can take anywhere from 5-10 min between incubation and freezing when using centrifugation, but that time decreases to 1-2 min using vacuum filtration with the most time-consuming step being transporting the samples from the bench to the freezer. Not only does this saved time cut down on transcriptional variation, but it also allows for more samples to be processed in the same amount of time increasing overall throughput for this method.

The second variation in this approach that differs from other methods is the use of zirconia/silica beads in combination with a lysis buffer for cell disruption as opposed to enzymatic or lysis buffer only methods. Mechanical cell disruption via bead beating has been shown to significantly increase RNA yields in *C. albicans*, compared to vertical vortexing without beads in lysis buffer alone (Rodríguez and Vaneechoutte, 2019). Zirconia/silica beads have a higher density than the typical glass beads used in bacterial (3.7 g/cm³ and 2.5 g/cm³ respectively) which increases their efficiency at rupturing the tough fungal cell walls. An additional benefit of mechanical cell disruption is that enzymatic methods such as zymolyase digestion have been shown previously to alter RNA expression profiles of the sample being assayed by activating stress response pathways thereby confounding the downstream analysis (Suzuki and Iwahashi, 2013).

Overall, by utilizing mechanical cell disruption and vacuum filtration combined with the commercially available Qiagen RNEasy MiniKit, this technique represents a fast, and efficient method for cell harvesting and RNA extraction saving time and giving more consistent and reliable transcriptional data.

Materials and Reagents

1. 15 ml polypropylene culture tubes (VWR, catalog number: 82050-274, item #187262)
Note: Can be from any source.
2. 25 ml serological pipettes (VWR, catalog number: 89130-912)
Note: Can be from any source.
3. 50 ml conical screw cap tube (Fisher Scientific, catalog number: 0553913)
Note: Can be from any source.
4. 1.5 ml screw cap tubes (Fisher Scientific, catalog number: 1415-8700)
Note: Can be from any source.
5. RNase-free 1.5 ml microcentrifuge tubes (Fisher Scientific, catalog number: 14-666-319)
Note: Can be from any source.
6. 250 ml 0.1 µm PES membrane vacuum filtration unit (Fisher Scientific, catalog number: 09741201)
Note: Can be from any source.
7. Cuvettes PS Semi-micro (VWR, catalog number 9700-586)
Note: This is dependent upon the method of measuring the OD₆₀₀ of cell cultures to be used.
8. Uvette 220-1600 nm (Eppendorf, catalog number: 952010051)
Note: This is dependent upon the method of quantifying purified RNA.
9. 0.5 mm zirconia/silica disruption beads (Research Products International, catalog number: 9834)

Follow manufacturer instructions to sterilize and eliminate nucleic acid contamination and store at -20 °C

10. MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane (Merk Millipore Ltd., MF-Millipore™, catalog number: HAWG04700)
11. Relevant *C. albicans* strains (SC5314 and an SC5314 derived *efg1Δ::HIS1* mutant strain constructed by the authors were used in this example)
12. Yeast extract (BD, Bacto™, catalog number: 212750)
13. Peptone (BD, Bacto™, catalog number: 211677)
14. Dextrose (Sigma Life Science, catalog number: D9434-1KG)
15. RPMI 1640 (Sigma-Aldrich, catalog number: R4130-10L), store at 4 °C
16. Fetal Bovine Serum Premium (Atlanta Biologicals, R&D Systems, catalog number: S11150H), store at -20 °C
17. Phenol:chloroform:isoamyl alcohol (Sigma Life Science, catalog number: 77617-100ml), store at 4 °C
18. Qiagen RNEasy Mini Kit (Qiagen, catalog number: 74104)
19. NaOH salt (Sigma-Aldrich, catalog number: 221465-500G)
Note: Can be from any source.
20. 100% pure Ethanol (Sigma-Aldrich, catalog number: E7023-1L)
Note: Can be from any source.
21. Deionized H₂O (any source)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: 444203-250mL)
Note: Can be from any source.
23. YPD liquid growth media (see Recipes)
24. 10 N NaOH (see Recipes)
25. RPMI + 10% FBS (see Recipes)

Equipment

1. Glass 125 ml Erlenmeyer flasks (Fisher Scientific, catalog number: FB501125)
Note: Can be from any source.
2. 30 °C incubator (any source)
3. Rotator drum for overnight cultures (New Brunswick Scientific, model: TC-7, catalog number: M1053-4004)
Note: This is out of production but can be substituted for a similar rotator that can reach 60-70 rpm.
4. Eppendorf BioPhotometer® D30 (Eppendorf, catalog number: 6133000010)
Note: This can be substituted for another spectrophotometer with similar capabilities.
5. Shaking incubator 37 °C (New Brunswick Scientific, Eppendorf, model: I-26, catalog number: M1324-0000)

Note: This can be substituted for another shaking incubator with similar capabilities.

6. Microcentrifuge (Thermo Scientific, model: Sorvall Legend Micro 21, catalog number: 75002436)
Note: This can be substituted for another microcentrifuge that can spin at $\geq 17,000 \times g$.

7. 1-2 L sidearm Erlenmeyer flask (any source)
8. MiniBeadBeater-16 (BioSpec Products, model: 607)

Note: The authors have not attempted this protocol with another type of bead beater, however a similar horizontal bead beater with similar specifications would most likely yield similar results.

9. Millipore 47 mm glass base and stopper (Millipore, catalog number: XX1014702)
10. 50 ml conical tube rack (any source)
11. -80 °C and -20 °C freezer (any source)
12. 4 °C refrigerator or cold room (any source)
13. Standard benchtop vortexer (any source)
14. Forceps

Procedure

Note: This procedure is written with volumes and quantities for two strains, mutant and Wild Type (WT), with three replicates each for a total of six independent samples.

D. Cell culture and harvesting

- a) Inoculate WT and mutant strains in 5 ml liquid YPD and incubate overnight at 30 °C with 60 rpm rotation.
- b) Pre-warm 200 ml RPMI + 10% FBS and empty sterile 125 ml Erlenmeyer flasks overnight (O/N) 37 °C, or for a minimum of 2 h prior to Step A3 with more time for larger volumes.
- c) Aliquot 25 ml pre-warmed RPMI + 10% FBS into each of the 6 pre-warmed 125 ml flasks and return to 37 °C incubator to stabilize temperature at least 90 min before first inoculation.
- d) Vortex O/N WT culture thoroughly, measure OD600 using Eppendorf BioPhotometer and Cuvettes PS Semi-micro, and inoculate first pre-warmed flask of RPMI + 10% FBS to a final OD600 of 0.2 from the O/N WT culture.
- e) Immediately transfer to I-26 air shaker incubator for 4 h at 37 °C and 225 rpm rotation. Replace O/C in 30 °C incubation.
- f) Wait 8-10 min between inoculation of replicates.

Note: This gap is to allow for adequate time for cell harvesting between replicates. Time can be shortened or lengthened as needed to allow for working speed of person performing protocol.

- g) Repeat Steps A4-A6 for WT replicates 2 and 3 as well as mutant replicates 1-3.
- h) This leaves roughly 3 h until the first culture is ready to harvest. Take this opportunity to set up cell harvesting equipment.
 - i. Connect 1-2 L sidearm flask to a vacuum line and trap and mount the Millipore 47 mm glass base and stopper for vacuum filtration in the top of the flask.
 - ii. Turn on vacuum and wash filter base 2x with dH₂O and 2x with 70% EtOH to eliminate

- debris and allow filter base to dry before applying filter membrane.
- iii. Chill 50 ml conical tube rack at -80 °C.
 - iv. Pre-chill and label 6 50 ml conical screw-cap tubes in ice.
 - i) Just before 4-hour timepoint apply MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane to filter base grid side up using sterile flat-blade forceps and turn on vacuum (Figure 1).



Figure 1. Cell harvest filtration setup. Millipore 47 mm glass base and stopper inserted into sidearm flask and connected to vacuum line. Wash glass base thoroughly with water and 70% ethanol with vacuum running and let dry. Place MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane onto glass base. In-line trap and filter are suggested to avoid carryover of waste.

- j) At 4 h, remove the WT replicate 1 flask from the shaker and swirl gently to dislodge cells stuck to the sides of the flask.
- k) Quickly, use a 25 ml serological pipette to transfer entire culture to filter membrane as fast as the vacuum will allow without overflowing the filter membrane.
- l) As soon as the liquid has been pulled off of the cells, transfer filter membrane to bottom of a pre-chilled 50 ml conical tube using sterile flat-blade forceps, and place immediately in -80 °C freezer.
- m) Clean the filter base as before with dH₂O and 70% EtOH.
- n) Repeat Steps A9-A13 for each of the remaining WT and mutant replicates.

Note: These steps are time sensitive. Shortening the time between when the cells are incubating to when they are frozen at -80 °C is critical for accurate expression data.

- o) Incubate cells at -80 °C for at least 1 h to ensure they are frozen before proceeding to RNA extraction.

Note: Cells are stable at this stage for several days to weeks, so if many samples are needed, cell culturing and harvesting can be split up over multiple days prior to RNA extraction.

E. RNA extraction and quantification

- a) Aliquot 300 µl 0.5 mm zirconia/silica disruption beads and 600 µl 25:24:1 phenol:chloroform:isoamyl alcohol to 1.5ml screw cap tubes and chill at 4 °C for at least 1 h (1 tube per cell culture replicate). Prepare fresh 600 µl RLT (Qiagen RNeasy Mini Kit) + 1% β-mercaptoethanol (BME) per sample and pre-chill in ice. Pre-chill 2 ml sterile dH₂O per sample on ice. Pre-chill 1.5 ml microcentrifuge tubes (1 per sample) on ice.

Note: Steps B2-B10 must be done at 4 °C. Keep materials on ice or work in a cold room.

- b) Remove 50 µl conical tube containing filter membrane and cells from -80 °C freezer and thaw on ice for 10 min.
- c) Wash cells off of filter membrane with 900 µl of chilled sterile dH₂O and vortex at top speed for 30 s. Transfer suspension to chilled 1.5 ml microcentrifuge tube and place on ice.
- d) Wash filter a second time with an additional 900 µl of chilled sterile dH₂O and vortex at top speed for 30 s. Transfer suspension to the same chilled 1.5 ml microcentrifuge tube and place on ice.
- e) Repeat Steps B3 and B4 for all samples using a new tube for each sample.
- f) Centrifuge in standard desktop microcentrifuge at max speed ($\geq 17,100 \times g$) for 30 s and discard supernatant.
- g) Add 600 µl prepared RLT + 1% BME solution and resuspend by vortexing at max speed.
- h) Transfer 600 µl of cell suspension to chilled screwcap tubes with zirconia beads and phenol:chloroform:isoamyl alcohol.
- i) Bead beat for 3 min at 4 °C using the BioSpec MiniBeadBeater-16 Model 607.
- j) Centrifuge cells at max speed for 8 min at 4 °C in standard desktop microcentrifuge.
- k) Transfer 550 µl of the aqueous layer to new RNase-free 1.5 ml microcentrifuge tube and add 550 µl 70% EtOH and mix by inverting 6 times.

Note: If there is not 550 µl of aqueous solution, measure and transfer to the new tube and add an equal volume of 70% EtOH.

- l) Transfer up to 700 µl of sample to RNeasy spin column and centrifuge for 15 s at $\geq 8,000 \times g$. Discard flow-through. Reload column with remaining sample and spin again discarding flow-through.

Note: All supplies referred to in Steps B12-B17 are provided in the Qiagen RNeasy Mini Kit Protocol for the Purification of Total RNA from Yeast. Steps B12-B16 of this protocol are the same as steps 2-5 of the Qiagen RNeasy Mini Kit protocol for Purification of Total RNA from Yeast.

- m) Add 700 µl Buffer RW1 to column and spin for 15 s at $\geq 8,000 \times g$. Discard flow-through.
- n) Add 500 µl Buffer RPE (with Ethanol added) to column and spin for 15 s at $\geq 8,000 \times g$. Discard flow-through.
- o) Repeat Step B14.

- p) Transfer spin column to a new collection tube and spin for 1 min at $\geq 8,000 \times g$. Discard flow-through.
- q) Transfer spin column to RNase-free 1.5 ml microcentrifuge tube (from kit). Add 40 μl RNase-free water to the column membrane and centrifuge for 1 min at $\geq 8,000 \times g$.
- r) Take 40 μl eluate from Step B17 and re-apply to the spin column membrane and spin again for 1 min at $\geq 8,000 \times g$.

Note: This is not strictly necessary but will increase overall concentration.

- s) Freeze samples immediately at -80 °C for storage.
- t) To quantify RNA and assess purity, prepare 1:500 dilution of total RNA and measure OD₂₆₀ using Eppendorf BioPhotometer and Uvette 220-1,600 nm cuvettes.

Notes:

- a. Average yield is typically over 2 $\mu g/\mu l$ up to around 6 $\mu g/\mu l$. 260/280 and 260/230 values typically range between 2.0-2.2 and 2.5-2.5 respectively.
- b. Dilution and use of Uvettes may not be necessary depending on equipment available.

Notes

1. This protocol is written to accommodate 6 samples (2 strains with 3 replicates each) but is scalable given that it can be stretched over multiple days while the harvested cells are frozen. It can also be adapted for whatever media conditions outside of RPMI + 10% FBS. However, with the addition of more replicates and strains it is recommended that a single large batch of media is made, if possible, ahead of time to eliminate batch effects on the transcriptional profile of the strains. For example, here we call for 200 ml of RPMI + 10% FBS for 6 samples which allows for 50ml extra. If 60 samples were needed instead, 1.5 L of RPMI + 10% FBS should be made up in one large batch then aliquoted into smaller units for use over several days.
2. This protocol does not include a DNase treatment. This means that the final product may have some DNA contamination. The Qiagen RNeasy Mini Kit spin columns prevent most DNA carry-over by design, but residual DNA may remain. If DNA contamination must be avoided for downstream applications, DNase treatment may be done on the spin column between Steps B12 and B13 or after final elution, and a protocol for this can be found in the Qiagen RNeasy Mini Kit protocol.

Recipes

1. YPD liquid growth media
 - a. Dissolve 20 g dextrose, 20 g peptone, and 10 g yeast extract with 500 ml dH₂O
 - b. Bring volume to 1 L with dH₂O
 - c. Autoclave to sterilize
2. 10 N NaOH

- a. Dissolve 40 g NaOH in 50 ml dH₂O
- b. Bring volume up to 100 ml with dH₂O
3. RPMI+10% FBS
 - a. Dissolve 3.28 g RPMI1640 powder in 180 ml dH₂O
 - b. Adjust pH to 7.4 using 10 N NaOH (approximately 180 µl)
 - c. Sterilize RPMI solution by vacuum filtration
 - d. Thaw Fetal Bovine Serum Premium (FBS) in 37 °C water bath

Note: The FBS manufacturer recommends avoiding repeated freeze thaw cycles, so plan accordingly.
 - e. Shake gently to resuspend any solids
 - f. Add 20 ml thawed FBS to 200 ml RPMI solution and mix with gentle shaking to avoid foaming
 - g. Store at 4 °C for up to a month

Acknowledgments

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Competing interests

The author declare that no competing interests exist.

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Colorimetric RT-LAMP Methods to Detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

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[Abstract] Standard diagnostic methods of Coronavirus Disease 2019 (COVID-19) rely on RT-qPCR technique which have limited point-of-care test (POCT) potential due to necessity of dedicated equipment and specialized personnel. LAMP, an isothermal nucleic acid amplification test (NAAT), is a promising technique that may substitute RT-qPCR for POCT of genomic materials. Here, we provide a protocol to perform reverse transcription LAMP targeting SARS-CoV-2. We adopted both real-time fluorescence detection and end-point colorimetric detection approaches. Our protocol would be useful for screening diagnosis of COVID-19 and be a baseline for development of improved POCT NAAT.

Keywords: SARS-CoV-2, COVID-19, LAMP, RT-LAMP, Colorimetric detection

[Background] Fast and sensitive detection of SARS-CoV-2, the etiologic agent of COVID-19, is important to control current pandemic situation as it enables early detection, isolation and treatment as well as monitoring screening. Current standard methods for detection of SARS-CoV-2 utilize RT-qPCR as World Health Organization recommends (WHO, 2020) However, proper performance of RT-qPCR diagnosis requires high profile facilities and specialists, often not available in the hospital/sampling places, thus, lacking POCT suitability.

Isothermal amplification methods are developed to accomplish diagnosis by nucleic acid detection in various point-of-care as it can be performed with relatively simple instruments (Niemz *et al.*, 2011). Loop-mediated isothermal amplification (LAMP) is one of such isothermal NAAT (Notomi *et al.*, 2000). Amplification by LAMP reaction can be observed through fluorescent dye with real-time PCR instruments (Oscorbin *et al.*, 2016), or through cost effective colorimetric detection methods (Goto *et al.*, 2009; Miyamoto *et al.*, 2015; Tanner *et al.*, 2015). Indeed reverse transcription LAMP (RT-LAMP) may be considered a promising tool as several other groups are employing this technique for SARS-CoV-2 detection (Yan *et al.*, 2020; Zhang *et al.*, 2020).

Here, we present our protocol used to develop RT-LAMP assays targeting SARS-CoV-2. Candidate primer sets are designed using web based tool PrimerExplorerV5 (<https://primerexplorer.jp/e/>) targeting SARS-CoV-2 specific regions compare to SARS-CoV and SARS-CoV-2 Nucleocapsid. The primer sets

are screened for limit of detection and threshold time measured through real time fluorescent method. Below illustrated protocol includes RNA standard preparation, titration of cultured viral RNA and LAMP reaction which are used during primer screening (Figure 1). Optimized RT-LAMP condition for finally selected two primer sets, namely “Nsp3_1-61” and “Nsp3_2-24”, are representatively provided. We also adapted leuco crystal violet (LCV) colorimetric detection method optimized for Bst 3.0 buffer system (Miyamoto *et al.*, 2015). A commercially available colorimetric RT-LAMP premix uses pH sensitive dye so that the premix is not compatible with viral genome extraction methods which affect pH of weakly buffered reaction solution (Lalli *et al.*, 2020). However, LCV method is compatible with such viral genome extraction methods because the color change depends on dsDNA product.

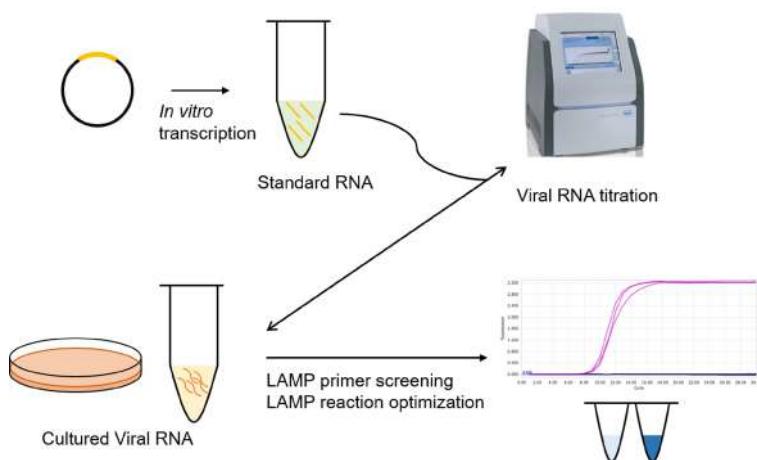


Figure 1. Protocol overview

Materials and Reagents

1. LightCycler® 8-Tube Strips, Clear (Roche, catalog number: 06327672001)
2. LightCycler® 480 Multiwell Plate 96, Clear (Roche, catalog number: 05102413001)
3. AccuPower PCR PreMix (-dye) kit (Bioneer, catalog number: K-2016)
4. Agarose (Bio Basic, catalog number: D0012)
5. 50x TAE (Biosesang, catalog number: T2002)
6. MEGAscript™ T7 Transcription Kit (Invitrogen, catalog number: AM1334)
7. Formaldehyde, 37% w/w aq. Soln. (Alfa Aesar, catalog number: A16163)
8. 10x MOPS (Bioneer, catalog number: C-9031)
9. SYBR™ Green II RNA Gel Stain (Invitrogen, catalog number: S7564)
10. RiboRuler Low Range RNA Ladder (Thermo Scientific, catalog number: SM1831)
11. QuantiFluor® RNA System (Promega, catalog number: E3310)
12. 1 M Tris-HCl, pH 7.5 (Biosesang, catalog number: T2016-7.5)
13. 0.5 M EDTA, pH 8 (Biosesang, catalog number: E2002)
14. QIAamp Viral RNA Mini Kit (Qiagen, catalog number: 52906)
15. Luna® Universal Probe One-Step Reaction Mix (NEB, catalog number: E3006)

16. DEPC treated water (Biosesang, catalog number: W2004)
17. WarmStart® Colorimetric LAMP 2x Master Mix (NEB, catalog number: E1800)
18. SYTO™ 9 (Invitrogen, catalog number: S34854)
19. Bst 3.0 DNA polymerase (NEB, catalog number: M0374, The product includes Isothermal Amplification Buffer II and Magnesium Sulfate solution.)
20. dNTP Mixture, 10 mM ea. (Enzyomics, catalog number: N002)
21. SuperScript™ IV Reverse Transcriptase (Invitrogen, catalog number: 18090050)
22. Crystal Violet (Sigma, catalog number: C0775)
23. Sodium Sulfite (Sigma, catalog number: S0505)
24. β-Cyclodextrin (Sigma, catalog number: C4767)
25. pET21a plasmid containing SARS-CoV-2 *Envelope* gene (Bionics, custom order)
26. T7 promoter primer (5'-AATACGACTCACTATAG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') (Macrogen) (Table 1)
27. Denaturing agarose gel (1%, 100 ml) (see Recipes)
28. 20 μM SYTO 9 (see Recipes)
29. 5x LCV solution (see Recipes)

Table 1. Primer sequences used for RT-qPCR and RT-LAMP

Primer set	Primer	Sequence (5'-3')	Note
E_Sarbeco	Forward	ACAGGTACGTTAACAGTTAACAGCGT	(Corman <i>et al.</i> , 2020)
	Reverse	ATATTGCAGCAGTACGCACACA	
	Probe	FAM-ACACTAGCCATCCTACTGCGCTTCG-BHQ1	
Nsp3_1-61	F3	GGAATTGGTGCCACTTC	
	B3	CTATTCACTTCAATAGTCTGAACA	
	FIP	CTTGGTACCAACAGTTGTTGACTTCAACCTGAAGAAGAGCAA	
	BIP	CGGCAGTGAGGACAATCAGACACTGGTGTAAGTTCCATCTC	
	LF	ATCATCATCTAACCAATCTTCTTC	
	LB	TCAAACAATTGTTGAGGTTCAACC	
Nsp3_2-24	F3	TGCAACTAATAAGGCCACG	
	B3	CGTCTTCTGTATGGTAGGATT	
	FIP	TCTGACTTCAGTACATCAAACGAATAAACCTGGTGATACGTTGTC	
	BIP	GACGCCAGGGATGGATAATTCCACTACTTCTCAGAGACT	
	LF	TGTTTCAACTGGTTTGCTCCA	
	LB	TCTTGCCTGCGAAGATCTAAAAC	

Equipment

1. Mupid-One (ADVANCE, catalog number: AD160)
2. ChemiDoc™ Touch Imaging System (Bio-Rad, catalog number: 1708370)

3. Quantus™ Fluorometer (Promega, catalog number: E6150)
4. LightCycler® 96 Instrument (Roche, catalog number: 05815916001)

Software

5. Polynucleotide Molecular Weight Calculator (Developed by Andrew Staroscik, <http://scienceprimer.com/nucleotide-molecular-weight-calculator>)
6. LightCycler® 96 Software (Roche)

Procedure

- F. Preparation of *in vitro* transcribed RNA standards for SARS-CoV-2 *E* gene
- a) Prepare PCR product as template for *in vitro* transcription: Run PCR with AccuPower® PCR PreMix (-dye) kit as manufacturer's instructions. Use pET21a plasmid containing SARS-CoV-2 *Envelope* gene as template and T7 promoter primer and T7 terminator primer.
 - b) Perform *in vitro* transcription using MEGAscript™ T7 Transcription Kit as manufacturer's instructions.
 - c) Confirm RNA product by subjecting a portion to formaldehyde-MOPS denaturing agarose gel electrophoresis. The same volume of RNA loading buffer II included in MEGAscript™ T7 Transcription Kit was added and heated for 10 min at 65 °C for sampling. Post-stain gel with 2x SYBR green II in 1x TAE and obtain image using ChemiDoc™ Touch Imaging System (Figure 2).

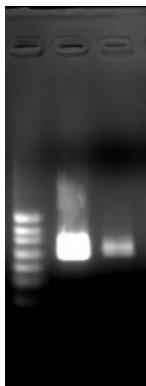


Figure 2. Agarose gel image of *in vitro* transcribed SARS-CoV-2 *E* gene RNA. Two 10-fold serial dilutions are loaded. Ladder sizes are corresponding to 1,000, 800, 600, 400, 300, and 200 bp from up.

- d) Measure RNA concentration with Quantus™ Fluorometer.
- e) Calculate RNA copy number.
 - i. Obtain molecular weight (M.W.) of the transcribed RNA (corresponding sequence: 5'-GGGGAAUUGUGAGCGGAUAACAUUCCCCUCUAGAAAUAUUUGUUUAACUUUA

AGAAGGAGAUUAUACAUUAUGUACUCUUUCGUUUCGGAAGAGACAGGUACGUUAAUA
GUUAAUAGCGUACUUCUUUUUCUUGCUUUCGUGGUUUUCUUGCUAGUUACACUAG
CCAUCUUACUGCGCUUCGAUUGUGUGCGUACUGCUGCAAUAUUGUUAACGUGAG
UCUUGUAAAACCUCUUUUUACGUUUACUCUCGUGUUAAAAACUGAAUUCUUCUA
GAGUUCUGAUCUUCUGGCUAACUCGAGCACCACCACCCACUGAGAUCC
GGCUGCUAACAAAGCCCCGAAAGGAAGCUGAGUUGGCUGCUGCCACCGCUGAGCAA
UAACUAGC-3') using Polynucleotide Molecular Weight Calculator, or using following formula where "#N" represents the number of each nucleotide.

$$\text{M.W.} = (\#A \times 329.2) + (\#U \times 306.2) + (\#C \times 305.2) + (\#G \times 345.2) + 159$$

- ii. Calculate copy number using following formula:

$$\text{RNA (copies}/\mu\text{l}) = \left(\frac{\text{concentration (ng}/\mu\text{l)}}{\text{molecular weight (g/mol)}} \times 10^{-9} \right) \times 6.022 \times 10^{23}$$

- f) Make 10-fold serial dilutions in TE buffer as RT-qPCR standards.

G. Viral RNA Preparation and Titration Using *E* gene

- a) Extract viral RNA from debris-free culture media with QIAamp Viral RNA Mini Kit following manufacturer's instructions.
Aliquot viral RNA extract and store at -80 °C until use.
- b) Titrate cultured viral RNA copy number by RT-qPCR
- i. Prepare primer mix as follows: for 100 µl, mix 10 µl of forward and reverse primer, 5 µl of probe, 75 µl of DEPC treated water. Stock concentration of each primer/probe is 100 µM. Names and sequences of primers are listed in Table 1. Primer mix may be prepared in a batch and stored in -20 °C for later use.
 - ii. Prepare master mix except template as follow: 7.5 µl of Luna® Universal Probe One-Step Reaction Mix, 0.75 µl of Luna® WarmStart® RT Enzyme Mix, 0.6 µl of primer mix, and 4.15 µl of DEPC treated water per reaction.
 - iii. Aliquot 13 µl of master mix to each well of PCR tube or plate.
 - iv. Add 2 µl of template (viral RNA dilutions, *in vitro* transcribed RNA standards or no template control) to each well. Close lid of PCR tube or cover film on plate.
 - v. Run PCR reaction as following temperature and time setting: 10 min at 55 °C, 1 min at 95 °C, and 45 cycles of 10 s at 95 °C and 30 s at 60 °C.
 - vi. Copy number of viral RNA can be calculated by substituting threshold cycle value (Ct) to the equation of standard curve of following form:

$$\text{Ct} = \text{slope} \times \log_{10}(\text{template copy number}) + (\text{y-intercept})$$

Standard curve generation and sample copy number calculation can be done using each software coupled with real-time PCR instrument.

H. RT-LAMP reaction

- a) Template viral RNA may prepared from cultured virus or biological specimen from COVID-19 patients. Selection of proper RNA extraction method is up to performers.
- b) Prepare 10x LAMP primer mix as follows: for 100 μ l, mix 16 μ l of FIP/BIP, 2 μ l of F3/B3 primers, 4 μ l of LF/LB primers and 56 μ l of DEPC treated water. Stock concentration of each primers is 100 μ M. Sequences of primers are listed in Table 1. The LAMP primer mix may be prepared in a batch and stored in -20 °C for later use.
- c) Prepare RT-LAMP reaction mix. Master mix except template can be prepared in a batch.
 - i. For LCV method, Reaction components, stock concentration and volume to use are as listed in Table 2.
 - ii. When using WarmStart® Colorimetric LAMP 2X Master Mix, prepare reaction mix as follows: 2 μ l of template, 1.5 μ l of 10x LAMP primer mix, 0.3 μ l of 20 μ M SYTO9, 7.5 μ l of LAMP mix, and 3.7 μ l of DEPC treated water.

Table 2. Components and volume used (μ l) for RT-LAMP with designated LAMP primer sets

Component	Nsp3_1-61	Nsp3_2-24
Template	2	2
10x LAMP primer mix	1.5	1.5
10x Isothermal Amplification Buffer II	1.5	1.5
MgSO ₄ (100 mM)	0.6	0.9
dNTPs (10 mM ea.)	1.5	2.1
5x LCV solution	3	3
SYTO 9 (20 μ M)	0.3	0.3
Bst 3.0 (8 U/ μ l)	0.75	0.75
SuperScript IV (200 U/ μ l)	0.1	0.1
DEPC treated water	3.75	2.85

- d) Aliquot 13 μ l of reaction mix and 2 μ l of template to each well of PCR tube or plate and close lid or cover film.
- e) Run isothermal amplification. Following example settings are for the LightCycler® 96 instrument SYTO 9 as fluorescent dye.
 - i. Select SYBR Green I and set “Integration Time” to 1 s.
 - ii. Set cycling condition to 59 s at 65 °C for Nsp3_1-61 primer set or 69 °C for Nsp3_2-24 primer set. Use 65 °C for reaction with WarmStart® Colorimetric LAMP 2x Master Mix. Set 59 s of incubation time and 1 s of data acquisition time for each cycle to make actual

incubation time per cycle as 1 min. Set number of cycles to the desired incubation time in minutes (Figure 3).

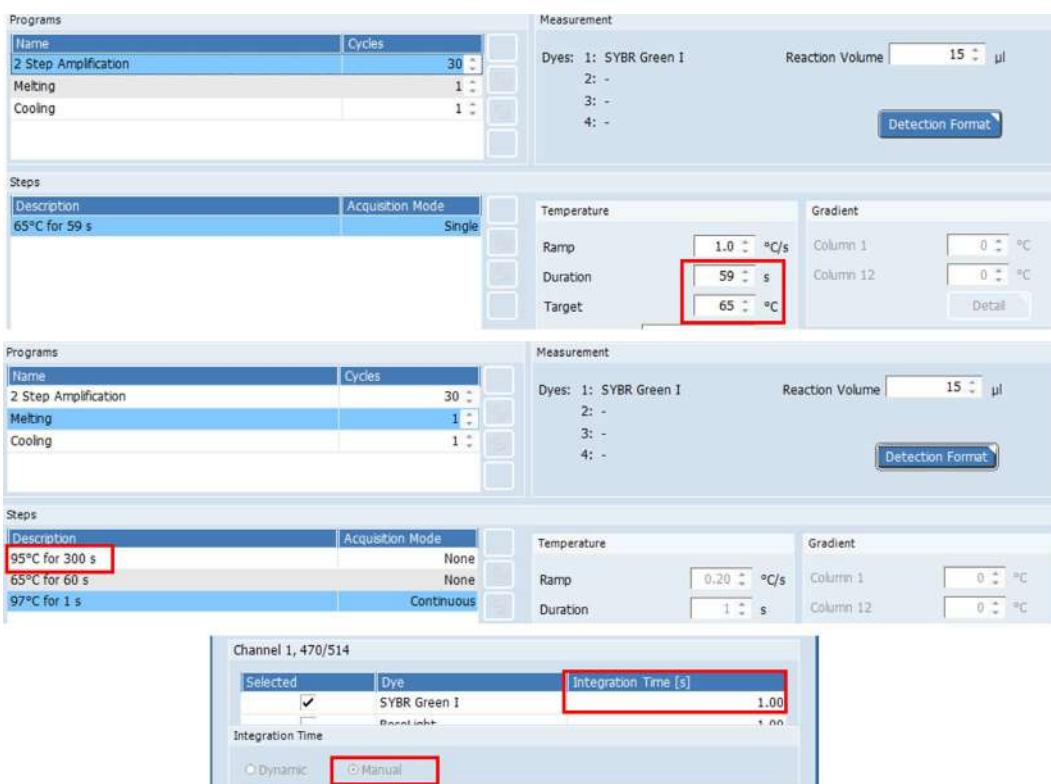


Figure 3. Example settings for the primer set Nsp3_1-61 using LightCycler® 96 software

Data analysis

1. Real-time amplification of LAMP reactions can be observed via LightCycler 96 software or other program accompanied with each qPCR machines. Sigmoidal amplification curve is shown for samples with positive result while no amplification is shown for negative samples or samples with negative results. Non-specific amplification curve may be observed with significantly delayed threshold time and/or shifted melting temperature (Figure 4).
2. For tests for which WarmStart Colorimetric LAMP 2x Master Mix is used, samples with positive result show yellow color while samples with negative result show pink color. For tests to which LCV method is adapted, samples with positive result show clear blue color from blueish transparent color (Figure 5).

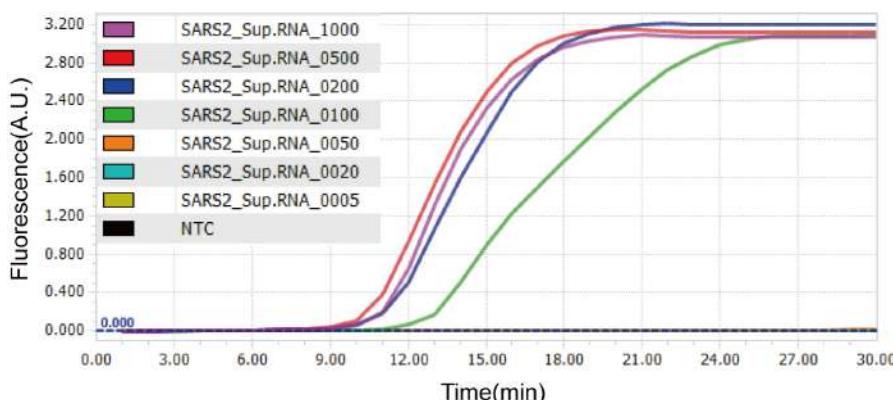


Figure 4. Amplification curves of RT-LAMP using Nsp3_1-61 primer set on LightCycler 96 system. Representative amplification curves of one replicate sample with designated RNA copies are shown. NTC, No Template Control.

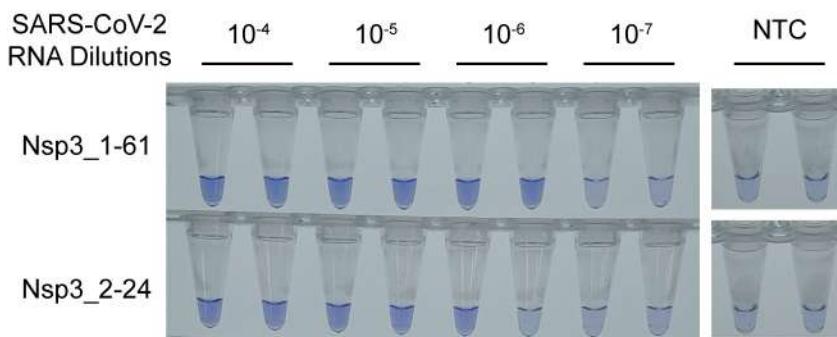


Figure 5. The color changes of LCV method for RT-LAMP. LCV color changes to blue by positive RT-LAMP amplification. RT-LAMP reaction was performed for 60 min at 69 °C with the half amount of SuperScript IV reverse transcriptase described in the main protocol as the result is from an experiment during reaction optimization. NTC, No Template Control.

Notes

1. Here we targeted *E* gene for RT-qPCR titration while LAMP primer targets *Nps3*. Therefore titrated RNA copy number does not perfectly match with LAMP target copy number.
2. Denaturing agarose gel for RNA electrophoresis should be prepared in fume hood as vapor with formaldehyde is toxic. It would be better to do electrophoresis in fume hood too.
3. *In vitro* transcription usually give micrograms of RNA products.
4. Same as or more than seven points with 5 to 10 fold serial dilution are generally recommended for standards of RT-qPCR. We used 10⁸ to 10⁰ copies per reaction and no template control (NTC) for standard curve generation. Triplicate for each dilution is sufficient.
5. While we obtained about 10⁷ copies/μl concentration from cultured virus, the number may differ by incubation period and virus species. Non-infected cells work as the negative control for viral infection yet the derived RNA is not necessarily included for RT-qPCR as no template control.

can serve as the negative control of RT-qPCR.

6. When preparing a master mix for RT-qPCR and LAMP/RT-LAMP in bulk for a batch of experiment, add extra volume to prevent shortage of the master mix from pipette error or other reasons. For 15 μ l reaction described in this protocol, 0.5 to 1 extra amount for < 20 reactions or about 10% extra amount for more reactions is usually enough.
7. For RT-qPCR and real-time RT-LAMP, other qPCR instruments than LightCycler® 96 Software can be used. For real-time RT-LAMP, the time required for measuring fluorescence should be accounted for setting incubation time and cycle scheme and for the calculation between Ct and threshold time. For example, Bio-Rad CFX systems need rather unpredictable data acquisition time as the system's detection unit scan each well one by one.
8. A melting step may be added to the RT-LAMP reaction since non-specific amplifications may be distinguished from their melting curves. Addition of an incubation step for 5 min at 95 °C between amplification and melting steps is rather recommended for polymerase inactivation.
9. When using LCV method for colorimetric detection, color varies by temperature. In high temperature, color of reaction mixture may turn to blue without nucleic acid amplification. Color of the mixture will back to normal in room temperature (18-25 °C) within 5 min.
10. The sensitivity of LAMP is bottlenecked by formation of 'dumbbell intermediate' and it is usually lower than that of PCR. However, LAMP is highly vulnerable to cross contamination because LAMP yield is high and the bottleneck is not applied. Therefore, we strongly discourage to open reaction tube after LAMP reaction. Otherwise, using separated space for analysis of reaction product (e.g., agarose gel electrophoresis) is recommended.
11. Typical LAMP optimization includes changing concentration of dNTPs or Mg²⁺. We suggest to test each concentration in combination because dNTPs can chelate Mg²⁺.
12. Since EDTA in TE buffer chelate Mg²⁺ ion, using DEPC treated water for RNA elution and dilution may be beneficial when testing larger volume of template.

Recipes

1. Denaturing agarose gel (1%, 100 ml)
1g agarose
10 ml 10x MOPS
18 ml 37% formaldehyde
72 ml DEPC treated water
2. 20 μ M SYTO 9
Dissolve 5 mM SYTO 9 original stock in DEPC treated water with 1/250 dilution. Aliquot in small volume (~100 μ l) and store at -20 °C
3. 5x LCV solution
10.20 mg Crystal Violet
378.12 mg Sodium Sulfite

283.75 mg β -Cyclodextrin
Dissolved in 50 ml DEPC treated water

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Competing interests

The authors declare no conflict of interests.

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Rapid Isolation and Purification of Secreted Bacteriocins from *Streptococcus mutans* and Other Lactic Acid Bacteria

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[Abstract] Bacteriocins are small ribosomally synthesized antimicrobial peptides produced by some microorganisms including lactic acid bacteria (LAB), a group of Gram-positive bacteria (cocci, rods) expressing high tolerance for low pH. Bacteriocins kill bacteria rapidly and are biologically active at very low concentrations. Bacteriocins produced by LAB are primarily active against closely related bacterial species. Many bacteriocins have been investigated with respect to their potential use in promoting human, plant, and animal health, and as food biopreservatives. Bacteriocins produced by LAB are particularly interesting since several LAB have been granted GRAS (Generally Recognized as Safe) status. Because it is not always possible to extract active bacteriocins secreted from cells grown in liquid medium, we developed a simple and inexpensive peptide extraction procedure using a semi-solid nutrient-rich agar medium. We hereby present a detailed procedure that leads to the rapid extraction of secreted bioactive bacteriocin peptides from the oral species *Streptococcus mutans*, a prolific bacteriocin-producing species, and its potential application for bacteriocin extraction from other LAB (e.g., *Streptococcus*, *Lactococcus*, *Enterococcus*). We also present a simple method for the detection of bacteriocin activity from the purified extracellular peptide extract.

Keywords: Bacteriocin, Extracellular peptide extraction, Lactic acid bacteria, Oral streptococci, *Streptococcus mutans*, Spot-on-Lawn assay, Bacteriocin activity

[Background] Most bacteria in nature do not exist independently but persist in complex multispecies biofilm communities (López et al., 2010). Numerous physical and nutritional interactions exist between bacteria contributing to the biofilm growth and survival. The production and secretion of bacteriocins to the extracellular space confer a distinct ecological advantage to the producer in competing against other bacteria that are present in the same ecological niche (Donia and Fischbach, 2015). Therefore, bacteria tightly regulate expression of bacteriocins to foster survival of the species within the biofilm.

The cariogenic organism *Streptococcus mutans* is found in the dental plaque, a biofilm community that forms on the tooth surface (Kolenbrander et al., 2010). Under normal circumstances, the dental plaque remains relatively stable (microbial homeostasis), contributing to dental health. However, a change in local environmental conditions can disrupt this natural homeostasis, leading to an ecological pressure that can cause disease. *S. mutans* is one of the most prolific producers of bacteriocins (referred to as mutacins) and bacteriocin production is considered to be an important factor in the colonization and establishment of *S. mutans* in the dental biofilm (Merritt and Qi, 2012). In our previous publications, we highlighted the role of *S. mutans* bacteriocins in the antagonistic interactions among oral streptococci

(Dufour and Lévesque, 2013; Dufour et al., 2020). We also showed that DNA uptake and bacteriocin production were controlled by a tightly regulated quorum sensing system suggesting that DNA exchange could be enhanced by bacteriocin secretion during the state of genetic competence (Perry et al., 2009a and 2009b; Dufour et al., 2011).

Most strains of *S. mutans* encode bacteriocins. Identification of putative bacteriocins can be easily done in silico by screening of genomic DNA sequences. For instance, the freely available software BAGEL4 (<http://bagel4.molgenrug.nl>) can be used for the detection of putative bacteriocin genes. It also analyzes the surrounding region on the bacterial genome for genes possibly encoding proteins involved in transport, immunity, and regulation. However, the putative bacteriocin loci identified in silico do not necessarily represent functional bacteriocin production. Consequently, bacteriocins must be extracted and purified in order to study them. Only few bacteriocins produced by *S. mutans* have been purified and characterized (Nicolas et al., 2007). This may be due to the many challenges associated with the production and extraction of these extracellular peptides. High variability of peptide recovery from one extraction to another is also usually observed. Since the volumetric bacteriocin production is dependent on the total biomass production, a nutrient-rich medium (e.g., animal tissue extracts) is also suggested for efficient production. In this protocol, we developed an extraction method from submerged cultures using a commercial medium supplemented with mineral salts and yeast extract (Dufour et al., 2020). This procedure has been successfully used by our group to extract biologically active bacteriocin peptides from oral streptococci (*S. mutans*, *S. salivarius*) isolated from the dental plaque biofilm of caries-free and caries-active subjects.

Materials and Reagents

39. Sterile 13 ml culture tube with ventilation cap (any type, e.g., Sarstedt, catalog number: 62.515.006)
40. Screw cap tube 50 ml (any type, e.g., Sarstedt, catalog number: 62.547.205)
41. Disposable glass Pasteur pipets 22.9 cm (9") (e.g., VWR, catalog number: 89061-526)
42. Disposable syringe, 50 ml (any type, e.g., Thermo Fisher Scientific, Fisherbrand, catalog number: 14-823-43)
43. Acrodisc® syringe filters with Supor® membrane 0.2 µm, 25 mm (Pall Corporation, catalog number: 4506)
44. Petri dishes with clear lid 100 x 15 mm (any type, e.g., Thermo Fisher Scientific, Fisherbrand, catalog number: FB0875712)
45. Aluminum foil (any product)
46. Bacteriocin-producing LAB (e.g., *Streptococcus*, *Lactococcus*, *Enterococcus*)
47. Bacterial target strain: *Micrococcus luteus* (e.g., strain ATCC 4698)
48. Dehydrated Todd-Hewitt broth (Thermo Fisher Scientific, BD Difco, catalog number: DF0492-17-6)

49. Dehydrated Columbia broth (Thermo Fisher Scientific, BD Difco, catalog number: DF0944-17-0)
50. Yeast extract (BioShop Life Science Products, catalog number: YEX401)
51. Agar (BioShop Life Science Products, catalog number: AGR001)
52. NaH₂PO₄ (e.g., Millipore Sigma, catalog number: S8282)
53. Na₂HPO₄ (e.g., Millipore Sigma, catalog number: S9763)
54. MgSO₄ (e.g., Millipore Sigma, catalog number: M7506)
55. FeSO₄ (e.g., Millipore Sigma, catalog number: F7002)
56. CaCO₃ (e.g., Millipore Sigma, Sigma-Aldrich, catalog number: C4830)
57. Chloroform ACS Reagent Grade (Caledon Laboratory Chemicals, catalog number: 3000-1-40)
58. Acetonitrile (for HPLC) (Thermo Fisher Scientific, Fisher Chemical, catalog number: A998)
59. Trifluoroacetic acid (Peptide and Protein Analysis/Certified) (Thermo Fisher Scientific, Fisher Chemical, catalog number: O4902)
60. Methanol (for HPLC) (Thermo Fisher Scientific, Fisher Chemical, catalog number: A452SK-4)
61. Hydrochloric acid solution, 6 N ACS (Certified) (Thermo Fisher Scientific, Fisher Chemical, catalog number: SA56-4)
62. Milli-Q water or any filtered ultra-pure water
63. THYE broth (500 ml) (see Recipes)
64. BAC semi-solid agar medium (2 L) (see Recipes)
65. Columbia-CaCO₃ agar plates (500 ml) (see Recipes)
66. Columbia soft agar (500 ml) (see Recipes)
67. ACN-TFA solvent (500 ml) (see Recipes)
68. Methanol:water, 95:5 v/v [pH 2] (500 ml) (see Recipes)
69. Methanol:water v/v gradient (500 ml) (see Recipes)

Equipment

1. Heavy-duty low form beaker 4 L (any type, e.g., VWR, catalog number: 10536-520)
2. Heavy-duty low form beaker 250 ml (any type, e.g., VWR, catalog number: 10536-390)
3. Scoop type spatula (any type, e.g., VWR International, catalog number: 470149-438)
4. Centrifuge bottles 250 ml (Thermo Fisher Scientific, Nalgene PPCO, catalog number: 3120-0250)
5. Reusable glass media bottle with cap 1 L (any type, e.g., Thermo Fisher Scientific, Fisherbrand, catalog number: FB8001000)
6. Reusable glass media bottle with cap 500 ml (any type, e.g., Thermo Fisher Scientific, Fisherbrand, catalog number: FB800500)
7. Sep-Pak® C18 environmental cartridges, 55-105 µm (Waters, catalog number: WAT023635)
8. CO₂ incubator (any type, e.g., Thermo Fisher Scientific, model: Hera Cell 150)
9. Water bath (any product)

10. Magnetic stirrer (any product)
11. Magnetic stir bar (any product)
12. Benchtop refrigerated centrifuge (any type, e.g., Eppendorf, model: 5810 R)
13. Floor refrigerated centrifuge (any type, e.g., Beckman Coulter, model: J2-21 M)
14. Ultra-low temperature freezer (any product, e.g., Thermo Fisher Scientific, model: Forma 900 Series)
15. SpeedVac vacuum concentrator (any product, e.g., Thermo Fisher Scientific, model: Savant SPD120)
16. Portable pump (any product, e.g., Bio-Rad, model: Econo Gradient Pump)
17. Bunsen burner
18. Cold (4 °C) lab chamber
19. Lab fume hood
20. Steam autoclave

Procedure

- I. Preparation of bacteriocin production medium and bacterial growth
5. Prepare the bacteriocin production BAC semi-solid agar medium in a beaker and keep the sterilized medium in the cold room overnight (18-24 h) before inoculation.
6. Prepare a preculture of bacteriocin-producing LAB strain of interest into a sterile 13 ml culture tube with ventilation cap containing 10 ml of THYE broth, and incubate statically at 37 °C with 5% CO₂ in a CO₂ incubator for 18 h.
7. Using a sterile glass Pasteur pipet, stab the BAC semi-solid agar medium approximately 300 times under aseptic conditions (Figure 1).



Figure 1. Stabbing of the bacteriocin production medium. Using a sterile glass Pasteur pipet of 22.9 cm, the semi-solid agar medium is stabbed multiple times. The depth of the stabs should correspond to the tip length of the pipet used (approx. 120 mm for a Pasteur pipet of 22.9 cm).

8. Pour the overnight preculture of the bacteriocin-producing LAB strain on the surface of the BAC semi-solid agar medium and cover the beaker with the same aluminum foil used for sterilization of the medium. Use 5 ml of an overnight preculture at an OD₆₀₀ of ~1.0 per liter of BAC semi-solid agar medium (Figure 2).



Figure 2. Inoculation of the bacteriocin production medium

9. Incubate the mixture at 37 °C with 5% CO₂ in a CO₂ incubator for 72 h (Figure 3).



Figure 3. Culture of bacteriocin-producing LAB obtained after 72 h of incubation. Bacterial growth of *Streptococcus mutans* LAB761, a strain encoding several bacteriocin loci, can be seen in stabbed BAC semi-solid agar medium.

J. Extraction of secreted bacteriocins

1. Mash the mixture using a spatula and transfer into 250 ml centrifuge bottles (Figure 4).

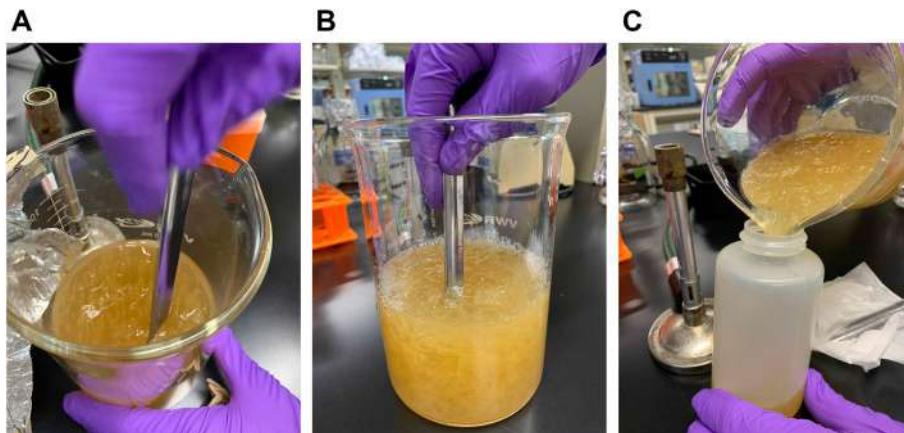


Figure 4. The stabbed bacteriocin medium showing bacterial growth (A) is mashed into small pieces using a spatula (B). The mashed mixture is then transferred into centrifuge bottles (C)

2. Store the bottles containing the mashed mixture in an ultra-low temperature freezer for 18 h.
3. Thaw the mixture by putting the bottles in a water bath at 65 °C for 1 h. The freeze/thaw cycle is necessary in order to disrupt the cell membrane of LAB.
4. Centrifuge at 15,000 $\times g$ for 40 min at 4 °C using a floor refrigerated centrifuge.
5. Carefully collect the supernatant (Figure 5A) and transfer into a 1 L glass bottle.
6. Add an equal volume of chloroform (Figure 5B) and shake vigorously by hand for 20 min (Figure 5C). Store the mixture at 4 °C overnight (18-24 h).

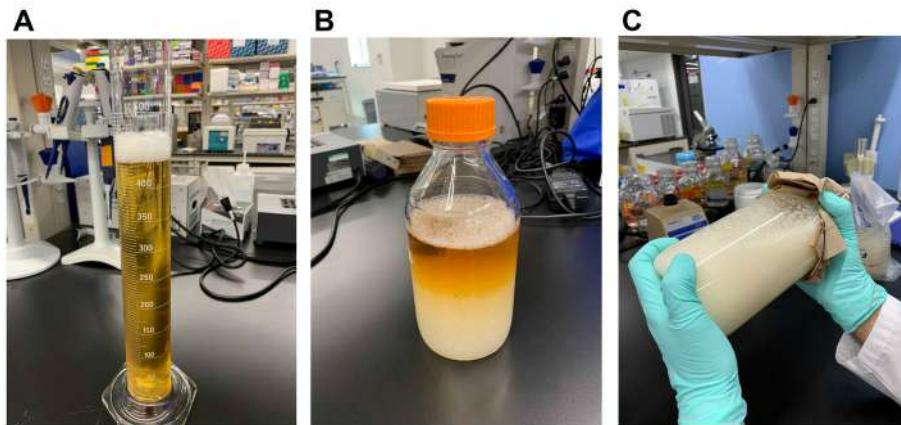


Figure 5. Chloroform extraction of bacteriocins. One volume of chloroform is added to one volume of collected supernatant (A). The mixture (B) is mixed thoroughly by hand for approx. 20 min (C).

7. Remove the upper layer and transfer the white layer into screw cap tube 50 ml (Figure 6). Let the emulsion settle on its own (approx. 5-10 min).

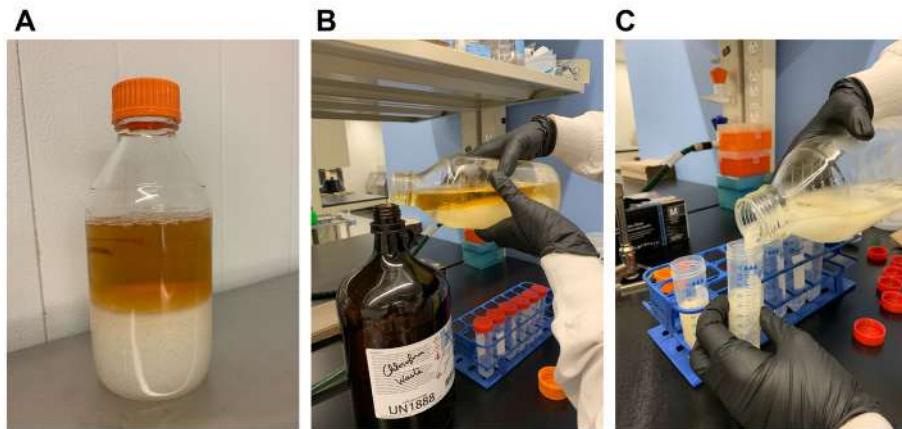


Figure 6. Bacteriocin extraction following chloroform method. The upper phase (A) corresponding to the “chloroform waste” is carefully removed (B) and the white material containing the secreted peptides is transferred into 50 ml tubes (C).

8. Collect the floating white interfacial layer and transfer into a glass beaker (Figure 7).

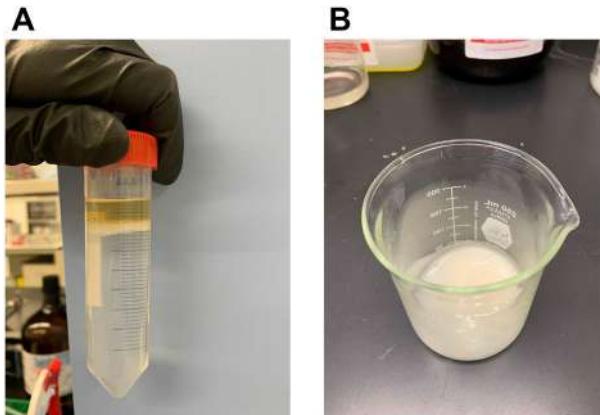


Figure 7. Extraction of bacteriocin following chloroform method. A floating white interfacial layer should be clearly visible (A). The white interface corresponding to the bacteriocin fraction is transferred into a glass beaker (B).

9. Keep the material in a fume hood until complete evaporation of the residual chloroform (approx. 2-3 days). A bacteriocin-containing powder of brownish color should be obtained (Figure 8).



Figure 8. Appearance of the extracellular peptide extract following complete evaporation

10. Dissolve the extracellular peptide extract in 25 ml of ACN-TFA solvent.
11. Transfer the resuspended extracellular peptide extract in a screw cap tube 50 ml.
12. Centrifuge at 3,200 $\times g$ for 40 min at 4 °C using a benchtop refrigerated centrifuge to remove insoluble materials.
13. Transfer the clear supernatant into a clean screw cap tube 50 ml.
14. The supernatant is passed through a 0.22 µm syringe filter with low protein binding membrane.
15. Prewash a C18 cartridge with 100 ml of methanol:water, 80:20 v/v.
16. The resultant liquid obtained at Step A18 is passed through a Sep-Pak® C18 cartridge using 50 ml of increasing concentration of methanol:water v/v (50%, 60%, 70%, 80%, and 95% [pH 2]) using a portable pump at a flow rate of 1 ml/min. All fractions are tested for bacteriocin activity using a spot-on-lawn assay.
17. To obtain a more concentrated peptide extract, dry the active fractions using a SpeedVac vacuum concentrator at room temperature and dissolve using ACN-TFA solvent to acquire desirable concentrations.
18. Keep an aliquot of the extracellular peptide extract at 4 °C for immediate use while the rest is stored in a freezer (-20 °C).

K. Detection of bacteriocin activity using spot-on-lawn

4. Prepare a preculture of a target strain into a sterile 13 ml culture tube with ventilation cap containing 3 ml of THYE broth, and incubate statically at 37 °C with 5% CO₂ in a CO₂ incubator for 18 h.
5. Measure the absorbance (OD₆₀₀) of the preculture and dilute the target strain in 4 ml of Columbia soft-agar pre-warmed at 55 °C to obtain a concentration of approximately 10⁷ CFU/ml using a plot standard curve (OD₆₀₀ vs. CFU/ml).
6. Pour the inoculated soft-agar onto the surface of a Columbia-CaCO₃ agar plate. Keep at room temperature until the top layer has solidified.

7. Prepare a series of two-fold serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) of the extracellular peptide extract in ACN-TFA solvent.
8. Drop 20 μ l aliquots of two-fold serially diluted extracellular peptide extract on top of Columbia-CaCO₃ agar plate overlay containing the sensitive target strain. Wait until the drops have been completely absorbed before incubating the plates. To accelerate the absorption, keep the plates next to the flame of a Bunsen burner with the lid slightly off for 10-15 min.
9. Incubate the plates at 37 °C with 5% CO₂ in a CO₂ incubator for 18 h.

Data analysis

1. Plates are visually examined for evidence of growth inhibition (Figure 9).



Figure 9. Antimicrobial activity of the extracellular peptide extract from *S. mutans* detected using the spot-on-lawn assay. Aliquots (20 μ l) of two-fold serially-diluted peptide extract (1/16, 1/32, 1/64 and 1/128 dilutions are shown) are tested.

2. Bacteriocin concentration is expressed as arbitrary unit (AU) per milliliter. AU is defined as the reciprocal of the highest dilution at which bacterial growth inhibition is visually detectable. Based on Figure 9, the bacteriocin concentration is estimated at 3,200 AU/ml (64 x [1,000 μ l/20 μ l]).

Recipes

1. THYE broth (500 ml)
 - a. In an autoclavable glass bottle (1 L) with cap, add 15 g of dehydrated Todd-Hewitt broth, 1.5 g of Yeast extract, and 400 ml of Milli-Q water
 - b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to completely dissolve
 - c. Adjust final volume to 500 ml with Milli-Q water
 - d. Autoclave on wet cycle at 121 °C for 30 min

Note: There is no need for pH adjustment. The cap must be loose to allow steam to escape. Broth can be stored at room temperature.
2. BAC semi-solid agar medium (2 L)
 - a. In a glass beaker (4 L), add 60 g of dehydrated Todd-Hewitt broth, 6 g of Yeast extract, 2 g

of NaH₂PO₄, 0.4 g of Na₂HPO₄, 1.4 g of MgSO₄, 0.01 g of FeSO₄, 6 g of agar, and 1.6 L of Milli-Q water

- b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to completely dissolve
- c. Adjust final volume to 2 L with Milli-Q water. Cover the beaker with aluminum foil
- d. Autoclave on wet cycle at 121 °C for 55 min

Note: There is no need for pH adjustment. The medium is kept at room temperature for 18 h before inoculation.

3. Columbia-CaCO₃ agar plates (500 ml)

- a. In an autoclavable glass bottle (1 L) with cap, add 17.5 g of dehydrated Columbia broth, 0.5 g CaCO₃, 7.5 g of agar, and 400 ml of Milli-Q water
- b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to dissolve
- c. Adjust final volume to 500 ml with Milli-Q water
- d. Autoclave on wet cycle at 121 °C for 30 min

Note: There is no need for pH adjustment. The cap must be loose to allow steam to escape. Although Columbia agar base powder can be purchased, we recommend to use the dehydrated Columbia broth and add agar separately. Lower bacteriocin activity was detected using Columbia agar base powder.

- e. Pour agar plates under aseptic conditions

Note: Precipitate of CaCO₃ will not dissolve completely. Agar plates are stored at 4 °C.

4. Columbia soft-agar (500 ml)

- a. In an autoclavable glass bottle (1 L) with cap, add 17.5 g of dehydrated Columbia broth, 3 g of agar, and 320 ml of Milli-Q water
- b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to completely dissolve
- c. Adjust final volume to 500 ml with Milli-Q water
- d. Autoclave on wet cycle at 121 °C for 30 min

Note: There is no need for pH adjustment. The cap must be loose to allow steam to escape. Soft-agar is kept at 55 °C and used the same day.

5. ACN-TFA solvent (500 ml)

- a. Prepare a solution of 35% acetonitrile-0.1% trifluoroacetic acid by diluting 175 ml of acetonitrile into 324.5 ml of Milli-Q water into a glass bottle (1 L) with cap
- b. Add 0.5 ml of trifluoroacetic acid
- c. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to mix

Note: The solvent is stored at room temperature.

6. Methanol:water, 95:5 v/v [pH 2] (500 ml)

- a. Prepare a solution of 95% methanol by diluting 475 ml of methanol into 25 ml of Milli-Q water into a glass bottle (1 L) with cap
- b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to mix
- c. Adjust the pH to 2.0 with HCl 6 N

Note: The solvent is stored at room temperature.

7. Methanol:water v/v gradient (500 ml)
 - a. Prepare a solution of 50%, 60%, 70% and 80% methanol by diluting 250 ml, 300 ml, 350 ml, or 400 ml of methanol into 250 ml, 200 ml, 150 ml, or 100 ml of Milli-Q water into a glass bottle (1 L) with cap, respectively
 - b. Place a magnetic stir bar in the bottle and stir using a magnetic stirrer to mix

Note: The solvents are stored at room temperature.

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Competing interests

The authors declare no competing interests.

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Generating Three-dimensional Human Granulomas *in vitro* to Study *Mycobacterium tuberculosis*-host Interaction

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[Abstract] Granulomas are organized multicellular structures that constitute the hallmark of an infection by the human pathogen *Mycobacterium tuberculosis* (*Mtb*). A better understanding of the complex host-*Mtb* interactions within the granuloma's environment may lead to new therapeutic or preventive tools to improve the control of the tuberculosis pandemic. To date, several *in vitro* models that are able to mimic human nascent granulomas have been reported. Here we describe a protocol in which *Mtb*-infected human peripheral blood mononuclear cells (PBMCs) are embedded within a collagen matrix leading to the formation of three-dimensional micro-granulomas. Subsequently, PBMCs and *Mtb* can be retrieved allowing multiparametric readouts from both the host and the pathogen. In addition to the incorporation of a physiological extracellular matrix, this model has the singular advantage of recapitulating dormant-like *Mtb* features, as well as reproducing *Mtb* resuscitation observed under immunomodulatory treatments, which have not been reported in other published protocols to generate *in vitro* granulomas.

Keywords: *Mycobacterium*, Tuberculosis, Granuloma, Host, *In vitro* model, Dormancy, Resuscitation

[Background] Tuberculosis (TB) is an air-borne disease that encompasses pulmonary and extra-pulmonary infections by the human pathogen *Mycobacterium tuberculosis* (*Mtb*). Causing an estimated 1.5 million deaths in 2019 (WHO, 2019), TB remains the world's deadliest infectious disease. The hallmark of the immunopathogenesis of TB is the formation of structurally organized, multicellular clusters called granulomas (Gengenbacher and Kaufmann, 2012). These structures mainly consist of a core of infected and non-infected macrophages surrounded by a rim of lymphocytes. The hostile environment within granulomas pushes *Mtb* to enter a slow- or non-replicating dormant state likely associated with a latent form of the disease. Consequently, *Mtb* dormancy leads to increased tolerance to antibiotics targeting metabolic pathways active during mycobacterial replication.

New strategies are urgently needed in order to lessen the TB death toll. Such strategies may arise from a better understanding of the infection within the granuloma's environment. However, the complex pathophysiology of *Mtb* infection makes the development of relevant preclinical models particularly challenging. Despite the widespread use of animal models in the TB field, the establishment of latency and granulomatous lesions comparable to the ones seen in humans can only be observed in non-human primates. Yet, drawbacks inherent to this animal model, such as its high cost or ethical concerns, have favored the development of *in vitro* models (Guirado and Schlesinger, 2013). In that context, several

independent groups have reported *in vitro* models based on the infection of human peripheral blood mononuclear cells (PBMCs) with virulent *Mtb* (Kapoor *et al.*, 2013; Guirado *et al.*, 2015; Agrawal *et al.*, 2016; Arbués *et al.*, 2020; Tezera *et al.*, 2017). Despite lacking some relevant cell types (e.g., neutrophils) and the full tissue conditions (such as lung structure or vascularization), such models are able to mimic the organization of human nascent granulomas, orchestrated by relevant cytokines.

The protocol described here notably incorporates two major advantageous features. On the one hand, *Mtb*-infected PBMCs are embedded in a three-dimensional (3D) matrix of collagen and fibronectin, main components of the lung extracellular matrix (Stek *et al.*, 2018). On the other hand, *Mtb* displays features associated with dormancy such as accumulation of intracellular triacylglycerides into lipid inclusions, loss of acid-fastness, transcriptional changes leading to a shift in carbon and energy metabolisms, and increase in antibiotic tolerance (Kapoor *et al.*, 2013). Remarkably, this model was also able to reproduce the differential rate of latent TB reactivation observed in the clinic upon treatment with different TNF- α -neutralizing biologics (Arbués *et al.*, 2020). Some particular limitations of this model to take into account are the difficulty of adding new immune cells for dynamic studies or its relatively low throughput.

In conclusion, the *in vitro* granuloma model described hereunder is particularly relevant for studies focusing on mycobacterial dormancy as well as further understanding the mechanisms involved in *Mtb* resuscitation.

Materials and Reagents

70. Filtered micropipette tips (various volumes) (e.g., Clearline, catalog numbers: 713115, 713117 and 713118)
71. Serological pipettes (various volumes) (e.g., Falcon, catalog numbers: 357543 and 357551)
72. 1.5/2.0 ml microtubes (e.g., Sarstedt, catalog numbers: 72.692.005 and 72.693.005)
73. 15/50 ml conical centrifuge tubes (e.g., Falcon, catalog numbers: 352096 and 352070)
74. Syringe (e.g., BD, catalog number: 300865)
75. 0.20 μ m syringe filter (e.g., Sarstedt, catalog number: 83.1826.001)
76. Stericup filter unit, Millipore Express PLUS high flow rate membrane (Millipore, catalog number: S2GPU01RE)
77. Tissue culture plates, 24-well, flat bottom (e.g., Falcon, catalog number: 353047)
78. Single-cell suspension of *Mycobacterium tuberculosis* (*Mtb*) prepared according to published methods (bacterial concentration determined by colony forming unit quantification)
Note: *Mtb H37Rv* (Sébastien Gagneux's strain collection) was used in our published study (Arbués *et al.*, 2020). Different strains or mycobacterial species could also be used.
79. Human peripheral blood mononuclear cells (PBMCs) isolated from whole blood or buffy coats following a standard protocol (Vogel *et al.*, 2018) (samples stored in liquid nitrogen can be used, when viability \geq 95% assessed by trypan blue exclusion)
80. RPMI-1640 medium, with L-glutamine and sodium bicarbonate (e.g., Sigma-Aldrich, catalog number: R8758)

81. Fetal bovine serum (FBS) (e.g., Gibco, catalog number: 10270), heat inactivated
82. Benzonase, *E. coli* recombinant (BioVision, catalog number: 7680)
83. Human serum off-the-clot, type AB (PAN Biotech, catalog number: P40-2701), heat inactivated and filtered (Stericup)
84. Trypan blue solution (e.g., Sigma-Aldrich, catalog number: T8154)
85. PureCol bovine type I collagen solution, 3 mg/ml (Advanced BioMatrix, catalog number: 5005)
86. Fibronectin from human plasma, 0.1% solution (Sigma-Aldrich, catalog number: F0895)
87. Dulbecco's Phosphate Buffered Saline (PBS) 10x (Sigma-Aldrich, catalog number: D1408)
88. Sodium hydroxide solution (NaOH) 1 N for cell culture (Sigma-Aldrich, catalog number: S2770)
89. Collagenase type IV, sterile filtered (Sigma-Aldrich, catalog number: C1889)
90. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (PanReac AppliChem, catalog number: A4689)
91. Triton X-100 (e.g., Sigma-Aldrich, catalog number: T8787)
92. Millipore double distilled water, sterile
93. Thawing medium (see Recipes)
94. Resting medium (see Recipes)
95. Complete medium (see Recipes)
96. Extracellular Matrix (ECM) solution (see Recipes)
97. Collagenase solutions (see Recipes)
98. 0.1% Triton X-100 (see Recipes)

Equipment

21. Class II biosafety cabinet
22. 5% CO₂ incubator, set at 37 °C
23. Cell counting chamber
24. Inverted bright-field/phase-contrast microscope (e.g., Leica DM IL LED)
Note: We routinely use a 20x objective to count the PBMCs and a 10x objective to monitor granuloma formation (Procedure A, Steps 1f and 2i, respectively)
25. Centrifuge for 15/50 ml conical tubes, maximum RCF ≥ 3,000 × g (e.g., Beckman Coulter Allegra X-15R)
26. Microcentrifuge for 1.5/2.0 ml microtubes, maximum RCF ≥ 6,000 × g (e.g., Eppendorf 5424R)

Procedure

*Note: This protocol was developed for infection with virulent Mtb; therefore, all work involving handling of Mtb should be performed in a biosafety level 3 laboratory under a class II biosafety cabinet. However, the protocol has also been successfully applied by our group to class 1 (*M. bovis* BCG) or class 2 (*Mtb* H37Ra) organisms in a standard biosafety level 2 laboratory.*

L. Generation of *Mtb*-induced 3D granulomas

10. Resting of PBMCs prior to infection

- a. Thaw one or two vials of PBMCs yielding the adequate amount of cells anticipated for the design of the experiment (2.5×10^6 PBMCs/well) and transfer them into conical centrifugation tubes.
- b. Wash the cells by adding 10 ml (the first 3-5 ml dropwise) of pre-warmed (37 °C) thawing medium and mix gently. Centrifuge for 5 min at $400 \times g$ and discard the supernatant by pipetting.
- c. Repeat this washing step.
- d. Resuspend the cell pellet in 5 ml of pre-warmed (37 °C) resting medium.
- e. Place the tube, with the lid loosened, into a 5% CO₂ incubator at 37 °C and allow the cells to rest for at least 6 h up to overnight.
- f. Count the rested PBMCs and assess cell viability by trypan blue dye exclusion method.

Note: Cell viability should be ≥ 95%. Lower cell viabilities can lead to Mtb-independent formation of cell aggregates.

- g. Transfer the required amount of PBMCs into a conical centrifuge tube. Pellet the cells by centrifugation at $400 \times g$ for 5 min and discard the supernatant by pipetting.
- h. Resuspend the PBMCs at 10^7 cells/ml in complete medium. Place the cells, with the lid of the tube loosened, into a 5% CO₂ incubator at 37 °C until use.

11. *Mtb* infection of PBMCs and embedding (Figure 1A)

- a. Prepare the required volume of extracellular matrix (ECM) solution according to Recipe 4, except for the 1 N NaOH. Keep at 4 °C/on ice until use.
- b. Infect the PBMCs with the volume of *Mtb* single-cell suspension required to reach the aimed multiplicity of infection (MOI). Mix thoroughly by pipetting up and down 10-15 times in order to obtain a homogeneous suspension of PBMCs and *Mtb*.

Note: Our experiments are routinely performed using a standard MOI of 1:200 (Mtb:PBMC). However, adjustments in the MOI may be required when using a new batch of single-cell suspension (or different mycobacterial strains or species). The optimal MOI can be experimentally assessed by monitoring the 3D granuloma formation in a dose-response setup (using various donors and uninfected PBMCs as a control).

- c. Distribute 250 µl per well of the *Mtb*-infected PBMCs in 24-well plates.
- d. Add the required amount of NaOH to the ECM solution (according to Recipe 4) and mix slowly and thoroughly by pipetting up and down 10-15 times.

Note: A thorough homogenization of the ECM solution after addition of NaOH is critical to reach the correct pH and, consequently, a proper setting of the matrix. If the NaOH is homogeneously incorporated into the ECM solution, its mixture with PBMCs (Step A2e) should result in a pale, yellowish pink color. However, if the ECM components are not properly mixed, a color gradient from dark pink to light yellow may be observed across the

wells due to pH variation. A too basic pH (dark pink) is detrimental for cell and bacterial viability and prevents the formation of granuloma-like structures.

- e. Add 250 µl of the ECM solution prepared in the previous step (1:1 ratio, v/v) to each well and mix by pipetting up and down 3-4 times.

- f. Place the plate into a 5% CO₂ incubator at 37 °C and allow the ECM to set for 45 min.

- g. Top up the set ECM with 500 µl per well of complete medium.

Note: If the full plate is not going to be used, fill up the surrounding empty wells with sterile PBS or water to minimize evaporation.

- h. Place the plate back into the 5% CO₂ incubator. Incubate at 37 °C for the desired time periods.

- i. Granuloma formation can be monitored using an inverted bright-field/phase-contrast microscope.

*Note: A marked donor-to-donor variability may be observed in terms of granuloma formation, in both number and size. This phenomenon has also been described in other *in vitro* granuloma models (Agrawal *et al.*, 2016).*



Figure 1. Schematic representation of the 3D *in vitro* granuloma model. A. *Mtb*-infected PBMCs are embedded in an extracellular matrix (ECM). Formation of granuloma-like structures can be observed after 7-8 days. B. PBMCs can be released from the ECM by collagenase digestion. C. Subsequent treatment with Triton X-100 allows the retrieval of *Mtb*.

M. Release of PBMCs from the ECM (Figure 1B)

1. At the selected time points, prepare the required volume of collagenase (1 mg/ml) according to Recipe 5.
2. Remove the supernatant from the wells to be processed. Discard, or filter sterilize and store at -80 °C if analysis of soluble factors is envisaged.
3. Add 250 µl per well of collagenase and incubate the plate in a 5% CO₂ incubator at 37 °C for 40-45 min. Every 15 min mix the liquid fraction by pipetting up and down to facilitate the digestion of the ECM.
4. Once the ECM liquefied completely, transfer the content of the wells into microtubes.
5. Pellet the cells by centrifugation at 400 x g for 5 min and discard the supernatant by pipetting.

6. Released cells can then be used to subsequently recover *Mtb* (according to Procedure C below), or to analyze host cell parameters, for example by flow cytometry (following standard staining procedures).

N. Retrieval of *Mtb* (Figure 1C)

1. Resuspend the cell pellet obtained in the previous section in 200 µl of 0.1% Triton X-100 by vortexing for a few seconds or pipetting up and down a few times.
2. Incubate for 20 min at room temperature to lyse the host cells.
3. To retrieve the *Mtb* pellet, centrifuge for 5 min at 6,000 x g and discard the supernatant by pipetting.
4. Various *Mtb* readouts can be obtained from the recovered bacilli as described in the Data analysis section.

Data analysis

Detailed information concerning data processing and analysis can be found in our original research article (Arbues *et al.*, 2020). Briefly, secreted cytokines can be measured by ELISA or multiplexed bead arrays from 0.2 µm-filtered supernatants. Host cell populations can be characterized by flow cytometry after staining and fixation of the released PBMCs. RNA analysis from both host and *Mtb* can be performed following standard procedures subsequently to TRI-reagent® treatment. *Mtb* load can be quantified by determination of colony forming units (CFU) on Middlebrook 7H11 agar supplemented with 10% OADC (0.05% oleic acid, 5% bovine albumin fraction V, 2% dextrose and 0.004% catalase). Importantly, dual auramine/Nile red staining can be used as a proxy of the metabolically active or dormant-like status of the recovered bacilli, respectively. As a general rule, we use at least three independent donors to generate every experimental readout.

Recipes

1. Thawing medium
RPMI supplemented with 10% FBS and 12.5 U/ml benzonase
2. Resting medium
RPMI supplemented with 10% FBS
3. Complete medium
RPMI supplemented with 20% human serum
4. Extracellular Matrix (ECM) solution
 - a. Recipe per ml:
 - i. 950 µl of collagen solution (3 mg/ml)
 - ii. 4 µl of human fibronectin (1 mg/ml)
 - iii. 50 µl of PBS 10x

- iv. 10 µl of 1 N NaOH
 - b. Calculate the volume of ECM solution required for the particular experiment to be performed:
number of wells to be embedded + ≥ 2 extra wells x 250 µl
 - c. On the day of the experiment, mix the collagen, fibronectin and 10x PBS and keep the mixture at 4 °C/on ice until use
 - d. Immediately before use, add the NaOH and mix slowly and thoroughly by pipetting up and down. The final pH of the solution should be ~7.0
5. Collagenase solutions
 - a. Collagenase buffer: PBS supplemented with 0.42 mM CaCl₂
 - i. Prepare a 0.5 M CaCl₂ solution (dissolve 1.47 g of CaCl₂·2H₂O in 20 ml of Milli-Q water) and sterilize (autoclave or filter)
 - ii. To prepare 50 ml of buffer, add 42 µl of 0.5 M CaCl₂ to 49.958 ml of PBS
 - b. Collagenase stock solution (10 mg/ml)
 - i. Dissolve 50 mg of sterile-filtered collagenase in 5 ml of collagenase buffer
 - ii. Prepare aliquots of convenient volume to prevent repeated freezing-thawing cycles and store at -20 °C (stable for several months)
 - c. Collagenase working solution (1 mg/ml)
 - i. Calculate the volume of diluted collagenase needed: number of wells to be processed + 1 extra well x 250 µl
 - ii. On the day of the experiment, dilute the collagenase stock tenfold in collagenase buffer to prepare the working solution (stable at 37 °C for at least 5 h)
 6. 0.1% Triton X-100
 - a. Prepare a 10% stock solution by mixing 5 ml of Triton X-100 with 45 ml of Milli-Q water
 - b. Filter sterilize the stock solution (stable at room temperature for several months)
 - c. To prepare 50 ml of working solution, add 500 µl of 10% stock to 49.5 ml of sterile Milli-Q water (stable at room temperature for several months)

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Competing interests

DP received the funding to implement this model from Novartis AG. The funders initiated the study design, but had no role in data collection and analysis. MK is a full-time employee of Novartis.

Ethics

Human PBMCs were isolated from buffy coats obtained from the Interregionale Blutspende SKR AG (Bern, Switzerland). All donors provided informed consent that included information on the use of blood products for research purposes.

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A Quick Method for Screening Biocontrol Efficacy of Bacterial Isolates Against Bacterial Wilt Pathogen *Ralstonia solanacearum* in Tomato

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[Abstract] *Ralstonia solanacearum* is a bacterial phytopathogen able to cause bacterial wilt disease in more than 200 plant species. Plant disease biocontrol strategies are used for controlling this disease and tomato is used as a model plant to conduct *R. solanacearum* associated studies. Conventional screening methods such as seed bacterization, soil drenching and root bacterization (in grown plants) to assess the ability of biocontrol bacteria to antagonize *R. solanacearum* under *in planta* conditions in different hosts are time-consuming and costly. A fast, cost effective method is a key requirement to advance the research on *R. solanacearum* biocontrol. In this protocol, we have inoculated the roots of tomato seedlings with bacterial isolates showing antagonistic activity against *R. solanacearum* under *in vitro* conditions. After 16 h of treatment with the antagonizing bacteria, seedlings were inoculated with *R. solanacearum* by a well-established root-dip method. Then the seedlings were maintained at controlled conditions and the number of wilted/dead seedlings were recorded up to 10th day post *R. solanacearum* inoculation. Biocontrol efficacy was calculated from the records for each tested isolate. This protocol is advantageous than already available protocols in the sense that it can be completed within a very short duration (~18 days for tomato) and there is no requirement of culture media to maintain the seedlings. This method can be used for quickly screening large number of bacterial isolates and different host genotypes within a short period of time and at a minimum cost.

Keywords: Bacterial wilt, *Ralstonia solanacearum*, Biocontrol agent, Biocontrol efficacy, Tomato

[Background] *Ralstonia solanacearum* is a soil-borne bacterial phytopathogen causing bacterial wilt disease in more than 200 crop species representing 50 different families (Seleim *et al.*, 2014), the majority from the family Solanaceae (Thera *et al.*, 2010). It can thrive up to several years in the soil retaining the capability to infect upon arrival of a host (Coutinho and Wingfield, 2017). Its natural route of entry is through the roots and finally, it ends up colonizing in the xylem vessels causing blockage of water conduit due to secreted exopolysaccharides with a corresponding wilting symptom observed in the leaves (Genin and Denny, 2012; Peeters *et al.*, 2013; Seleim *et al.*, 2014). Despite having usual practices of disease control such as the use of resistant plant varieties, industrial pesticides (Yuliar *et al.*, 2015), the use of biocontrol agents is a much-preferred choice in the current scenario due to environment friendly nature of the biocontrol agents. Bacteria and bacteriophages have been reported as biocontrol agents against *R. solanacearum*, of which bacteria being the predominant (Yuliar *et al.*, 2015). The assay for the potential to antagonize *R. solanacearum* under *in vitro* conditions is quite easy

as standard methods like agar well diffusion (Balouiri *et al.*, 2016), disc diffusion (Gupta *et al.*, 2015) and spot-on lawn assay (Vijayakumar and Muriana, 2015) are available. To be considered as a biocontrol agent of practical use it must primarily have the ability to control the pathogen under *in planta* condition and it should not have any negative effect on other living beings. The ability of self-sustainability and neutral effect on other living beings is a subject of later consideration, the first and foremost being its ability to antagonize the pathogen under *in planta* condition (Wang *et al.*, 2019). Conventional methods of bacterial biocontrol agent screening against *R. solanacearum* under *in planta* conditions such as seed bacterization (Siddiqui and Meon, 2009) and soil drenching (Van Elsas and Heijnen, 1990) have several limitations as they require viz. 1) large amount of inoculum, 2) prolonged maintenance of plants and 3) large space. In general, these demerits sum up these methods as time consuming and costly. To further advance the research on biocontrol bacteria against *R. solanacearum*, a quick screening method escaping the limitations of above-mentioned methods is required. Considering the entry of this pathogen through the roots, it was hypothesized that the application of biocontrol bacteria in the root would have the maximum chance to antagonize *R. solanacearum* thereby attenuating or eliminating its disease-causing capacity. The method described here is easy to perform and economically viable as it requires very less resource and time. The method is useful to screen biocontrol efficacy of several bacterial isolates simultaneously against *R. solanacearum* in a very limited space.

Materials and Reagents

1. Micro centrifuge tube 1.5 ml (Tarsons, catalog number: 500010)
2. Petri dish 90 mm (Tarson, catalog number: 460050)
3. Petri dish 60 mm (Tarson, catalog number: 460061)
4. Microtips 200-1,000 µl (Tarson, catalog number: 521020)
5. Microtips 2-200 µl (Tarson, catalog number: 521010)
6. Microtips 0.2-10 µl (Tarson, catalog number: 521000)
7. Absorbent cotton wool (Bengal Surgicals Limited, India/Azpack, catalog number: 12356477)
8. Tissue paper roll (Solimo/Kimberly-Clark WypAll L10 Paper Wipes, catalog number: 13478218)
9. Polygrid micro tube stand (Tarson, catalog number: 205110)
10. Plastic tray (Agrawal Plastic and Packaging Industries)
11. Cotton plugs (Prepared in laboratory)
12. Spray bottle (Abdos, catalog number: P11191)
13. Inoculating loop (Himedia, catalog number: LA014)
14. Hand gloves (Kimtech, catalog number: 97613)
15. Spatula (Himedia, catalog number: LA007)
16. Parafilm (Himedia, catalog number: LA017)
17. HiDispo™ Bag-12 (Himedia, catalog number: PW040)
18. Tomato seeds var. Pusa Ruby (JAI Kisan Seeds Private Limited, India)

19. *R. solanacearum* antagonizing bacteria (*Staphylococcus warneri* GL1, *Bacillus velezensis* GL3, *B. velezensis* GL5 and *B. velezensis* GMC2) isolated in authors laboratory from the endosphere of *Gnetum gnemon*
20. *R. solanacearum* F1C1 strain (provided by Prof. S.K. Ray, Tezpur university)
21. 70% ethanol (Himedia, catalog number: MB106-500ML)
22. 1% NaOCl (Himedia, catalog number: AS102-100ML)
23. Sterile distilled water
24. Peptone (Himedia, catalog number: RM001-500G)
25. Acicase (Himedia, catalog number: CR013-500G)
26. Glucose (Himedia, catalog number: MB037-500G)
27. Nutrient broth (Himedia, catalog number: M002-500G)
28. Glycerol (Himedia, catalog number: AS100-500ML)
29. Agar powder (Himedia, catalog number: CR301-500G)
30. Casamino acid peptone glucose broth (see Recipes)

Equipment

1. Beaker (Borosil, catalog number: BRL_1060D21)
2. Erlenmeyer flask 250 ml (Borosil, catalog number: 4980021)
3. Glass beaker (Borosil, catalog number: 1000D121)
4. Forceps (Tarsons, catalog number: 486000)
5. Spirit lamp/alcohol burner lamp (Himedia, catalog number: LA275)
6. Growth Chamber (Hipoint, model: 740FHLED)
7. Orbital shaking incubator (Remi, model: CIS-24 plus)
8. Refrigerated centrifuge (Sigma model: 2-16KL)
9. Autoclave (Optics, model: 50 litres)
10. Water purification system (Sartorius, model: Arium mini Plus UV)
11. Pipettor (Eppendorf Research Plus, catalog numbers: 3120000020, 3120000046, 3120000062)
12. Weighing balance (Shimadzu, model: BL220H)
13. Laminar air flow cabin (Cleanair Systems, model: CAH 1200)
14. Eppendorf Biophotometer (Eppendorf, model: D30)

Software

1. SPSS 20.0
2. Microsoft Office Excel 2016

Procedure

A. Tomato seedling preparation

1. Surface sterilize the tomato seeds by treating with 70% ethanol for 5 min followed by 1% NaOCl for 5 min and five washes with sterile water under laminar air flow cabin.
2. Take the seeds in a glass beaker with sterile water, stir gently and wait for 10 min.
3. Discard the seeds floating on surface of water after 10 min to maximize the number of potentially viable seeds.
4. Keep the beaker with the remaining seeds at 28 °C, in the dark for 24 h.
5. Wash the soaked seeds three times with sterile water.
6. Prepare a three-layered germination bed—sterile tissue paper on top, 3 cm thick sterile cotton in the middle and a polythene layering (HiDispoTM Bag) at the bottom. Place this bed in a plastic tray (30 × 20 cm²) and soak the bed with sterile water (Video 1).



Video 1. How to prepare germination bed

7. Spread the seeds uniformly on the bed and place this germination tray in a growth chamber conditioned at 28 °C, 75% relative humidity and 12 h of photoperiod (light intensity 450 µmol m⁻² s⁻¹).
8. Spray sterile water on a regular basis to sustain the growth of seedlings.
9. Five days old seedlings are used for priming with *R. solanacearum* antagonizing bacterium.

B. Antagonistic bacterial inoculum preparation

1. Culture the *R. solanacearum* antagonizing bacterium individually in nutrient broth medium at 28 ± 2 °C in shaking condition (120 rpm) for 24 h in the dark.
2. Centrifuge the culture broth at 430 × g for 10 min at room temperature and discard the supernatant. Resuspend the cell pellet in sterile water without vortexing.
3. Repeat the above step two more times and adjust the cell density of final suspension to ~10⁸

CFU ml⁻¹ (OD = 0.4). About 10 ml *R. solanacearum* antagonizing bacterial cell suspension is sufficient to prime 30 seedlings.

C. Seedling root bacterization with antagonistic bacterium

1. Uproot 5 days old tomato seedlings from the germination tray without damaging the root system.
2. Put 30 seedlings in a Petri dish (60 mm diameter, 10 mm height) with 10 ml of *R. solanacearum* antagonizing bacterial cell suspension. Ensure the contact of the root with the bacterial suspension (Figure 1).



Figure 1. Seedling root bacterization

3. Keep the Petri dish with seedlings in a growth chamber conditioned at 28 °C, 75% relative humidity, and 12 h of photoperiod for 16 h (light intensity 450 µmol m⁻² s⁻¹).
4. Treat 30 seedlings with sterile water to serve as a negative control group, which will be inoculated later with *R. solanacearum* (CK+R in Table 1).
5. Mock inoculate another 30 seedlings with sterile water to act as control group to nullify the natural death of seedlings in hydroponic conditions without being exposed to any bacteria (CK+W in Table 1).
6. After 16 h of priming with antagonizing bacteria, seedlings are ready for *R. solanacearum* inoculation.

Table 1. The types of treatment groups required in this method with description and purpose

Group	Treatment	Purpose
Control group-1 (CK+W)	Neither inoculation with antagonizing bacterium nor <i>R. solanacearum</i>	To nullify the effect of naturally wilted/died seedlings in the treatment groups. Seedlings should not wilt/die in this group.
Control group-2 (CK+R)	Mock inoculation with sterile water followed by <i>R. solanacearum</i> inoculation	To act as a negative control group. All or most of the seedlings should wilt/die.
Test group (GL1+R, GL3+R, GL5+R, GMC2+R)	Inoculation with antagonizing bacterium followed by <i>R. solanacearum</i> inoculation	To calculate biocontrol efficacy of antagonizing bacterium. Number of wilted/died seedlings will depend on the biocontrol efficacy of the antagonizing bacterium being tested.

D. *R. solanacearum* inoculum preparation

1. Culture *R. solanacearum* in casamino acid-peptone-glucose (CPG) broth at 28 ± 2 °C in shaking condition (120 rpm) for 24 h in the dark.
2. Centrifuge the culture broth at $430 \times g$ for 10 min at room temperature and discard the supernatant. Resuspend the cell pellet in sterile water without vortexing.
3. Repeat the above step two more times and adjust the cell density of final suspension to $\sim 10^8$ CFU ml⁻¹.

E. Inoculation of *R. solanacearum* in antagonistic bacteria primed seedlings

1. Take out the seedlings from the Petri dish and keep them in another sterile, dry Petri dish before *R. solanacearum* inoculation. Use these seedlings for inoculation immediately within 5 min, otherwise seedlings may dry out.
2. Inoculate the seedlings with *R. solanacearum* by root-dip inoculation method described by Singh *et al.* (2018). Briefly, take the *R. solanacearum* suspension in a wide mouth tube and immerse the seedling in the inoculum upto the root shoot junction for a few seconds.
3. Seedlings of the group CK+W should not be inoculated with *R. solanacearum*. It is kept as control.
4. Place the inoculated seedlings in 1.5 ml microcentrifuge tube and then add 1 ml sterile water into it. Use one seedling per microcentrifuge tube.
5. Place the microcentrifuge tube in a rack and keep inside a growth chamber conditioned at 28 °C, 75% relative humidity, and 12 h of photoperiod for 10 days (light intensity 450 µmol m⁻² s⁻¹). Add an equal amount of sterile water to the tubes on a regular basis to sustain the viability of seedlings.
6. A seedling is considered wilted when the leaves turn yellow to dark brown and the shoot bends at a sharp angle along the side of the microcentrifuge tube (Figure 2).

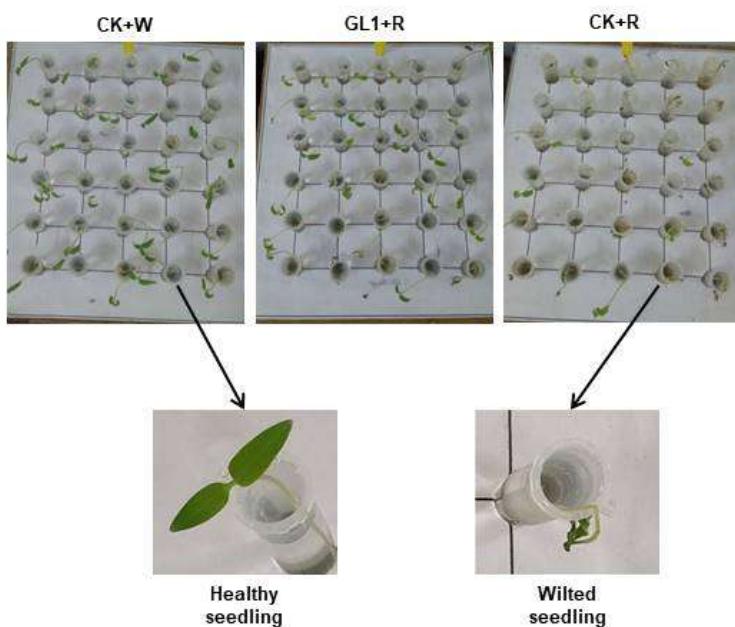


Figure 2. Effect of different treatment on tomato seedlings after 7 days of *R. solanacearum* inoculation. CK+W: neither inoculation with antagonizing bacterium nor *R. solanacearum*, GL1+R: inoculation with antagonizing bacterium *Staphylococcus warneri* GL1 followed by *R. solanacearum* inoculation, and CK+R: mock inoculation with sterile water followed by *R. solanacearum* inoculation. This figure is reprinted (with slight modification) from original research article Agarwal *et al.* (2020) with permission from Elsevier.

7. Keep records of wilted seedlings on a daily basis from 1st day to 10th day post *R. solanacearum* inoculation at an interval of 24 h.
8. A stepwise demonstration of the protocol is given in Figure 3.

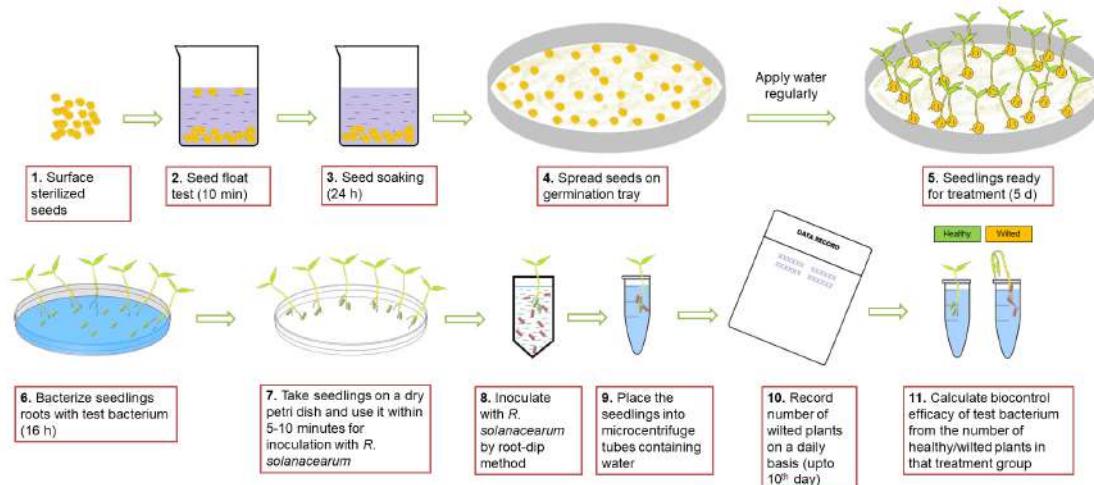


Figure 3. Schematic diagram representing biocontrol efficacy assay for *R. solanacearum* antagonizing bacteria

F. Calculation of biocontrol efficacy

1. Calculate disease severity index (DSI) using the formula:

$$\text{DSI} = \left(\frac{\text{no. of diseased plants}}{\text{total number of plants inspected}} \right) \times 100\%$$

2. Calculate biocontrol efficacy (BE) using the formula:

$$\text{BE} = \left(\frac{(\text{DSI of } R. solanacearum \text{ treated control} - \text{DSI of antagonist treated group})}{\text{DSI of } R. solanacearum \text{ treated control}} \right) \times 100\%$$

3. Calculate the DSI in the groups, and BE of antagonizing bacterium from the records on 10th day post *R. solanacearum* inoculation.

Data analysis

1. Perform One-Way ANOVA on the calculated DSI and BE, with *P* value less than 0.05, between-treatments in order to test the performances of biocontrol bacteria in the treatment groups.
2. To further investigate the nature of the differences between the control and different treatment means, perform Duncan Multiple Range Test (DMRT) after a statistically significant ANOVA.
3. Perform descriptive statistics (mean and standard error) on the calculated DSI and BE across all the treatment groups.
4. Data (Figure 4) can be found in the original research article titled “Endophytes from *Gnetum gnemon* L. can protect seedlings against the infection of phytopathogenic bacterium *Ralstonia solanacearum* as well as promote plant growth in tomato”; doi:10.1016/j.micres.2020.126503, published in Microbiological Research (Agarwal *et al.*, 2020).

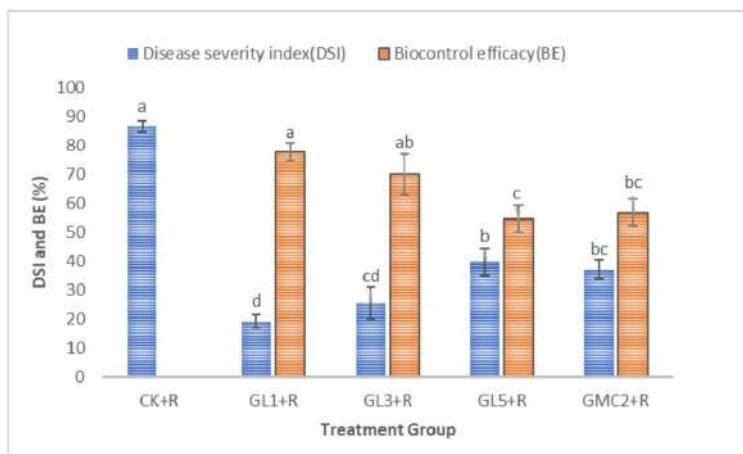


Figure 4. Biocontrol efficacy of bacterial isolates and respective disease severity index in treatment groups. CK+R: only *R. solanacearum* treated control, GL1+R: *Staphylococcus warneri* GL1+R. *R. solanacearum*, GL3+R: *Bacillus velezensis* GL3+R. *R. solanacearum*, GL5+R: *B.*

velezensis GL5+*R. solanacearum*, GMC2+R: *B. velezensis* GMC2+*R. solanacearum*. Different lower-case letters represent significant differences between treatments (one-way ANOVA, $P < 0.05$) according to Duncan's multiple range tests. This figure is reprinted (with slight modification) from original research article Agarwal *et al.* (2020) with permission from Elsevier.

Notes

1. Confirm the antagonistic ability of bacterial isolate against *R. solanacearum* by any of the standard methods such as agar well diffusion, disc diffusion, or spot on lawn assay with at least three repetitions prior to start this *in planta* biocontrol efficacy assay.
2. Calculate the age of seedlings from the day of putting the seeds on the germination tray.
3. The culture media, and incubating conditions for optimal growth can be different for different *R. solanacearum* antagonizing bacteria. This change will not have any effect further, since the cell number is adjusted for all isolates and media contamination is removed from the bacterial cells after repeated wash with sterile water.
4. In the initial root bacterization stage, take some additional seedlings in order to compensate any damage/death of seedlings while handling.
5. The number of seedlings in treatment groups can be increased as per convenience and to ensure the biocontrol efficacy of bacterial isolates, repeat this assay at least three times.
6. This assay cannot be conducted for more than 10 days post *R. solanacearum* inoculation in tomato, as seedlings (control group-1, Table 1) are unable to sustain after this time period in sterile water without any supplements. The age up to which the seedlings can sustain in sterile water is a subject of consideration before trying this assay on other hosts.

Recipes

1. Casamino acid peptone glucose broth (pH 6.5-7.0) (100 ml)
100 mg Acicase
1 g Peptone
500 mg Glucose
Add distilled water up to 100 ml volume and autoclave at 121 °C under 15 psi pressure for 20 min

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Competing interests

The authors declare no conflict of interests.

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Long-distance Transport in Bacterial Swarms Revealed by Single Nanoparticle Tracking

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[Abstract] During swarming, high density flagella-driven bacteria migrate collectively in a swirling pattern on wet agar surfaces, immersed in a thin viscous fluid layer called “swarm fluid”. Though the fluid environment has essential role in the emergence of swarming behavior, the microscopic mechanisms of it in mediating the cooperation of bacteria populations are not fully understood. Here, instead of micro-sized tracers used in previous research, we use gold nanorods as single particle tracers to probe the dynamics of the swarm fluid. This protocol includes five major parts: (1) the culture of swarming bacterial colony; (2) the preparations of gold nanorod tracers and the micro-spraying technique which are used to put the nanotracers into the upper fluid of bacterial swarms; (3) imaging and tracking; (4) other necessary control experiments; (5) data analysis and fitting of physical models. With this method, the nano-sized tracers could move long distances above motile cells without direct collisions with the bacteria bodies. In this way, the microscopic dynamics of the swarm fluid could be tracked with high spatiotemporal resolution. Moreover, the comprehensive analysis of multi-particle trajectories provides systematic visualization of the fluid dynamics. The method is promising to probe the fluid dynamics of other natural or artificial active matter systems.

Keywords: Collective motion, Active matter, Bacteria swarming, Gold nanorods, Single particle tracking

[Background] Recently, collective motions of bacteria have attracted a lot of attention in the field of active matter/fluid (Rabani *et al.*, 2013; Zhang, H. *et al.*, 2010; Marchetti *et al.*, 2013). Due to the self-driven motion of individual bacterium propelled by flagella, high density bacteria suspensions would fall into a non-equilibrium state and emerge collective structures such as vortices, jets (Mendelson *et al.*, 1999) or turbulences (Wensink *et al.*, 2012) in a scale far larger than the size of an individual bacterium. As one of the most widely used model system for the study of bacteria collection motions (Copeland and Weibel, 2009), bacteria swarming refers to coordinated migrations of cells across wet solid agar surfaces with swirling patterns (Kearns, 2010). During swarming, motile cells are immersed in a thin layer of highly viscous fluid called “swarm fluid” (Wu and Berg, 2012). Physically, the interplay between the fluid medium and the stirring flagella provides the dynamic origins of the collective motion though far-field hydrodynamic interactions (Koch and Subramanian, 2011). Biologically, the fluid environment enhances the mixing and diffusion of oxygen, nutrition as well as the signaling molecules involved in Quorum sensing (Hardman *et al.*, 1998) and chemotaxis (Taktikos *et al.*, 2012). Although the swarm fluid is important for the generation of large-scale synergetic patterns, how it works and its relationships with fast moving cells remain unclear especially at the microscopic scale.

Single particle tracking has been regarded as a powerful technique to investigate the complex fluid

(Burov *et al.*, 2011). Previous studies have used micro-sized particles as single particle tracers to characterize the properties of swarm fluid. Wu *et al.* used 1~2 μm micro-bubbles produced by explosive transformation of the water insoluble surfactant Span-83 droplets as tracers to reveal the intensive matter transfer flow in the leading edge of the swarming colony (Wu *et al.*, 2011). Zhang *et al.* fabricated MgO particles as tracer particles through burning magnesium ribbons. They found that 0.2 μm MgO particles only diffused normally within a small region ($\sim 4 \mu\text{m}^2$) as the swarm front approaches (Zhang, R. *et al.*, 2010). However, these methods of producing tracer particles are complicated and difficult to control. In addition, the prepared gold nanorods are often uneven in size. On the other hand, micro-sized tracers with various sizes and materials would either inevitably collide with bacteria bodies or be trapped at the liquid-air surface, making the tracers' motions incapable of revealing real dynamics of the fluid motions. Plasmonic gold nanorods (AuNRs) have long been used as nanotracers for long-duration observations in biological studies with high spatio-temporal resolution. The AuNRs have good photostability and low cytotoxicity. Moreover, the methods of preparing stable, uniform, and monodispersed AuNRs are very mature. Here, with *Bacillus subtilis* as a model strain, we introduced 40 \times 84 nm AuNRs as tracers into the bacterial swarm fluid. The AuNRs tracers could move freely in the upper layer without obvious physical contacts with the cell bodies. Combined with high spatiotemporal resolution of imaging and tracking technique, the trajectories of multi-nanoparticles in the large field of view could be obtained. By detailed diffusion analysis of tracers' trajectories, we found that the swarm fluid could transport the AuNRs to long distances in a super diffusive, Lévy mode (Figure 1). This result is consistent with previous studies that individual bacterium in swarming would exhibit characteristic motions of Lévy walk (Ariel *et al.*, 2015).

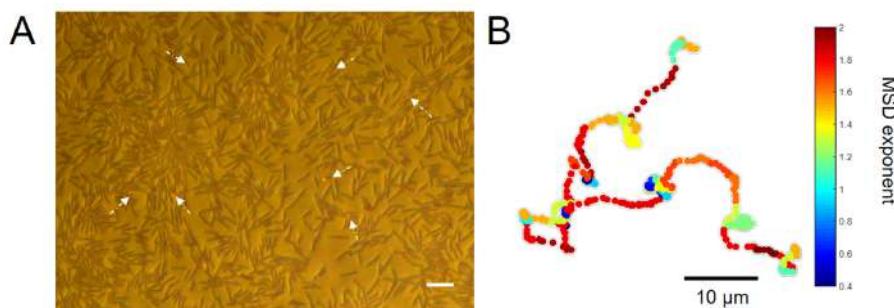


Figure 1. The gold nanorods move in high-efficiency on the upper fluid layer of the swarming bacteria. A. A captured image of the swarming bacteria and AuNRs. The brown rods are bacteria. The red spots which are indicated by white arrows are AuNRs. The scale bar is 10 μm . B. A typical trajectory illustrates that AuNRs move in a super-diffusive, Lévy mode with heterogeneous MSD local exponents.

Materials and Reagents

99. Plastic Petri dishes, 60 mm and 90 mm. Brand is not critical
100. Serum bottles, 250 and 500 ml (brand is not critical)

Note: Ensure that the bottles could withstand temperatures of at least 121 °C.

101. Square cover glass slides, 22 x 22 mm (Corning, catalog number: 2870-25)
102. Single concave microscope glass slide, 25.4 x 76.2 mm (Sail Brand, catalog number: CAT.NO.7101)
103. Disposable sterile syringe filters, 0.22 µm diameter (Bioland, catalog number: PT13-022)
104. 230 mesh carbon film, copper grids (Zhongjing Keyi, catalog number: BZ110223b)
105. *Bacillus subtilis* NCIB 3610 (purchased from China General Microbiological Culture Collection center, catalog number: 1.3358)
106. Tryptone, Bacto (BD Difco, catalog number: 211705)
107. Yeast extract, LD, Bacto (BD Difco, catalog number: 210933)
108. Bacto Agar (BD Difco, catalog number: 214010)
109. NaCl (Beijing Chemical Works, catalog number: A1060005)
110. LB agar, powder (Solarbio, catalog number: L1015, storage: 2-8 °C)
111. SH-PEG modified gold nanorods 40 x 84 nm (NanoSeedz, catalog number: PEG-40-650-50)
112. COOH-modified polystyrene microspheres, 0.5 µm (Dae technique, catalog number: PSC-00500)
113. Gold nanospheres 90 nm and 120 nm

Note: We synthesized the gold nanospheres according to the methods in the reference paper (Zhou et al., 2011).
114. Milli-Q H₂O (from Milli-Q Water Purification System, Millipore Sigma)
115. Soft LB agar for swarming (see Recipes)
116. LB broth, solution (see Recipes)

Equipment

15. Micro pipettes, 5, 10, 100, 200 and 1,000 µl (Eppendorf)
16. Autoclave (purchased from Alibaba) (brand is not critical)
17. Microwave oven (Meidi) (brand is not critical)
18. 4 °C and -80 °C refrigerator
19. Shaker incubator (Jiecheng Experimental Apparatus, model: TS-100B)
20. Constant temperature and humidity incubator (Yiheng Instrument, model: LHS-80HC-II)
21. UV-visible spectrophotometer (Shimadzu Corporation, model: UV1800)
22. Hand ultraviolet ray examining lamp, 365 nm (Yuhua Instrument, model: ZF-7) (brand is not critical)
23. High-frequency vibrating atomizer, 5 W, 5 V (Purchase from Alibaba, SKG, catalog number: 37288557693)
24. Inverted microscope equipped with a 100 W halogen tungsten lamp, a multipurpose dry condenser and polarizer/analyzer combination (Nikon, model: Ellipse Ti-U)
25. Long working distance objective, CFI TU Plan FLUOR BD 20x, NA/WD 0.45/4.5 mm (Nikon)

26. 20x objective, Plan Fluor 20x, NA/WD 0.5/ 2.1 mm (Nikon)
27. Upright metallographic microscope equipped with a 100 W halogen tungsten lamp, and an Epi illuminator containing an orthogonal polarizer/analyzer module (Nikon, model: LV100D)
28. High-precision piezo-Z positioning stage (PZ-2150)
29. Color CMOS camera (Olympus, model: DP74)
30. Upright dark-field microscope with a 100 W halogen tungsten lamp and a dark-field oil condenser (Nikon, model: 80i)
31. Inverted phase contrast microscope (Olympus, model: CKX53)

Software

3. ImageJ (An open source software written in Java, <https://imagej.nih.gov/ij/>)
4. Matlab (The MathWorks, Inc., <https://www.mathworks.com/>)
5. Multi-particle tracking software (self-developed in MATLAB, Github: <https://github.com/threebullets/ParDet>)
6. Origin 8 (OriginLab, <https://www.originlab.com/>)
7. PIVlab-particle image velocimetry (PIV) tool (William Thielicke, 2014) (GUI based tool developed by William Thielicke, <https://pivlab.blogspot.com/>)
8. SPSS (IBM, <https://www.ibm.com/analytics/spss-statistics-software/>)

Procedure

G. Swarming bacteria culture

12. Prepare the LB broth, Solarbio LB agar and soft LB agar culture solution according to the **Recipes**. First, add weighed reagents into 300 ml Milli-Q H₂O until completely dissolved. After that, put the dissolved culture solution into the autoclave under 121 °C, 20 min. Next put them in the microwave until boiling. Finally place them in the 4 °C refrigerator for later use.

Note: While putting the glass bottles into the autoclave, for safety make sure the caps are not too tight. In addition, the prepared culture medium has a shelf-life even if stored in the refrigerator. Stop use it if you find that it cannot maintain the quality as before.

13. Prepare the soft agar plates for bacterial swarming. First boil the soft LB agar solution to 100 °C in the microwave oven. After cooling down to 60 °C, add 3 ml solution to the 60 mm Petri dishes. Continue to cool down in the ultra-clean workbench until the liquid solidifies into the gel state.

14. Prepare the solid agar plates with Solarbio LB agar solution for bacterial strain activation and transmission. The procedures are like Step A2.

Note: The flatness, hardness as well as the wetness of the agar surface have great influences on the growth and the movements of bacterial colonies. In addition, the thickness of the agar plates is essential for the bacteria culture. For the LB solid agar plates, the thickness is usually 2/3 height of the Petri dishes. For the soft agar plates, we need ensure that the thickness of soft

agar plates fits the working distance of the objectives. In addition, the degree of transparency of agar is an important factor affecting microscopic observation.

15. To revive the frozen bacteria strains, dissolve the purchased *B. subtilis* 3610 powder which were sealed in a vacuum glass vial in the proper amount of LB broth solution and put it into the bacterial culture shaker under 37 °C, 200 rpm overnight. Then incubate the next generation on the Solarbio LB agar with streaking method using transfer needles.
16. To obtain the swarming bacterial colony, 5 µl of the bacteria overnight culture is inoculated at the center of the soft agar plates. Then the plates are placed in the laminar flow bench air drying for 5-8 min. After that, store it in the incubator at 30 °C and at least 90 %rh humidity for about 2-4 h.

Note: Make sure the humidity in the incubator are more than 90 %rh. Because the bacterial colony would not exhibit collective swarming behavior in the environment without enough humidity.

H. Preparation of AuNR solutions

4. To examine uniformity of the size and shapes of the particles, measure the UV-Vis spectrum of the SH-PEG AuNRs to characterize the positions of plasmonic resonance absorption peaks and acquire the transmission electron microscope images of the SH-PEG AuNRs. There exist two peaks in the UV spectrum. One is located around 650 nm and the other is around 522 nm. Under electron microscopy, AuNRs should be rod-shaped and uniform in size, with an average size of 40 × 84 nm.

Note: To prepare AuNRs samples for TEM examination, pipette 5 µl of undiluted AuNRs (145.36 µg/ml) and drop it on the copper grid. Evaporate naturally at room temperature. Repeat 2 or 3 times.

5. Dilute the SH-PEG AuNR solutions (145.36 µg/ml) for 20-fold with Milli-Q H₂O.

I. Micro-spraying technique

1. Put the AuNRs tracers into the upper surface of swarming bacteria fluid. First add 50 µl of the diluted AuNRs solution on the high-frequency vibrating atomizer.
2. Spray the atomized AuNRs aerosol out in a direction parallel to the surface of the swarming colony.

Note: The spraying direction and the distance to the surface of the colony should be adjusted to avoid the damages to the collective moving bacteria. In general, the atomizer are placed about 8 cm above the Petri dish. The direction of the spraying is preferably parallel to the colony surface or slightly inclined so that the aerosol could fall naturally onto the surface of the colony. Avoid spraying vertically to the colony.

J. Imaging

1. Examine the moving states of the bacterial colony using phase contrast microscope under 20x

or 40x objectives.

2. The area of the swarming bacterial colony will continuously expand with the number of bacteria increasing gradually. In the lag phase, the bacteria cells remain stationary. After a lag time of about 2 h, we can observe that the bacteria near the edge of the colony begin to swarm actively in swirls and jets. Once the bacteria start swarming, immediately take the specimen to the inverted microscope (Figure 2A).

Note: Special imaging dishes are not needed.

3. Tune the positions of the dark-field condenser and the working distance of the 20x objectives (Figure 2B) until the red color AuNRs and brown color bacteria could be imaged simultaneously.
4. Capture the images for 40 s with a frame rate of 60 fps using DP74 color CMOS camera (Figure 2C) in RGB channel under dark-field mode. Three parallel experiments are required.

Note: The environmental temperature and humidity (30 °C, 90 %rh) should be maintained just as in the incubator during the process of imaging. In addition, the intensity of light source of the microscope should not be too high to avoid the photodamages to the specimens. Damage to the cells would cease the collective movements of bacteria.

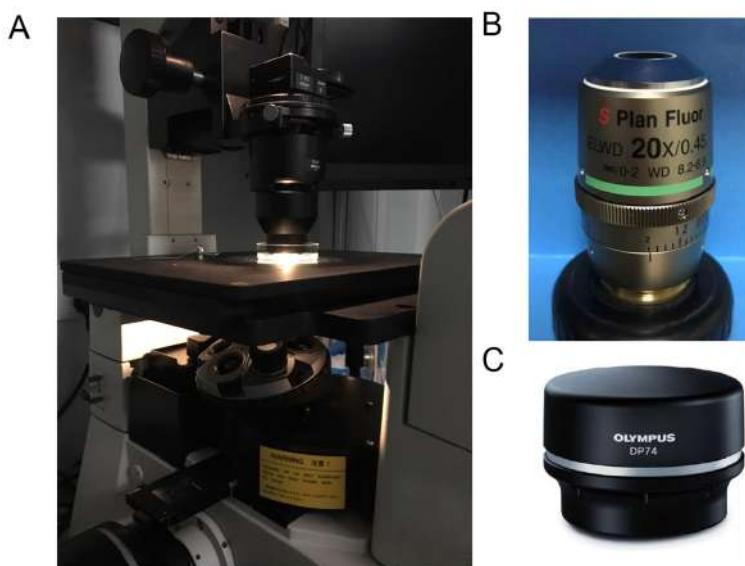


Figure 2. Experimental setups for imaging. A. The inverted Nikon Eclipse Ti-U microscope. The specimens are placed under a dark-field air condenser. B. 20x long working distance objective. C. The Olympus DP74 color camera.

K. Localize the z-axis position of AuNRs

1. Measure the distance between the bacteria layer and the AuNRs layer under the cross-polarization mode of the upright metallographic microscope.
2. Capture the time-lapsed images of the focal plane of the bacteria layer and the AuNRs layer respectively. Record their z-axis positions.
3. Record the 3D images of optical slicing starting from the bacteria layer to the AuNRs layer

through tuning the piezo-Z positioning stage. The z-axis resolution of the piezo-Z positioning stage is 0.01 μm . The z-increment steps typically chosen are about 0.2 μm .

4. Repeat the experiments in at least three different colonies.

Note: More elaborate procedures could be found in the section of “transparent methods”-3. Cross polarization microscopy for distance measurements between the AuNRs layer and the bacteria layer” of original paper.

L. Other control experiments

1. To obtain the motions of 90 and 120 nm gold nanospheres on the upper fluid of swarming bacteria, use the same tracing and imaging method as AuNRs particles.
2. To obtain the motions of PS spheres on the upper fluid of swarming bacteria, use the same tracing and imaging method as AuNRs particles.
3. To obtain the motions of AuNRs in the suspensions which are filtered out of bacteria, first filter the cells from the cultured bacteria suspensions with a 0.22 μm diameter syringe filter. Then pipette the 50-fold diluted AuNRs solution to the single concave glass slide. After covering the cover slides, take the specimen to the upright dark-field microscope equipped with 20x Nikon objectives. Then acquire the images with DP74 color CMOS camera.
4. To obtain the motions of AuNRs in the non-swarming bacterial colony without swarming, irradiate the swarming bacterial colony with a UV lamp for at least 10 h until the collective motions completely stops. Then spray the AuNRs aerosols as the procedures described in Procedure C. Observe the colony under Nikon LV 100D microscope in the reflective dark-field mode.

Data analysis

A. PIV analysis

Perform the flow field analysis of bacteria swarming on PIVlab software according to the steps below.

The work flow is also shown in the Figure 3A (a-f) in detail.

1. Run PIVlabGui.m in MATLAB.
2. Load and import images. Generally, choose the 1-2, 2-3, 3-4 sequencing style unless there are other requirements.
3. Set analysis parameters by Analyses settings > PIV settings > Pass 1 > Pass 2...4. Set the PIV algorithm to the FFT window. Define the size of interrogation areas and the steps in pixels.
Note: Use the options for simple image pre-processing if necessary, such as selection of ROI in “Exclusions” and noise reduction by “Enable CLAHE”, “Enable highpass” and other denoise filters.
4. Run PIV analysis by Analysis > Analyze! > Analyze current frame > Analyze all frames.
5. Refine velocity limits by Post processing > Vector validation.
6. Plot PIV images though Plot > Derive parameters/ modify data.

7. Export the txt file, videos, and images Figure 3B by File > save.

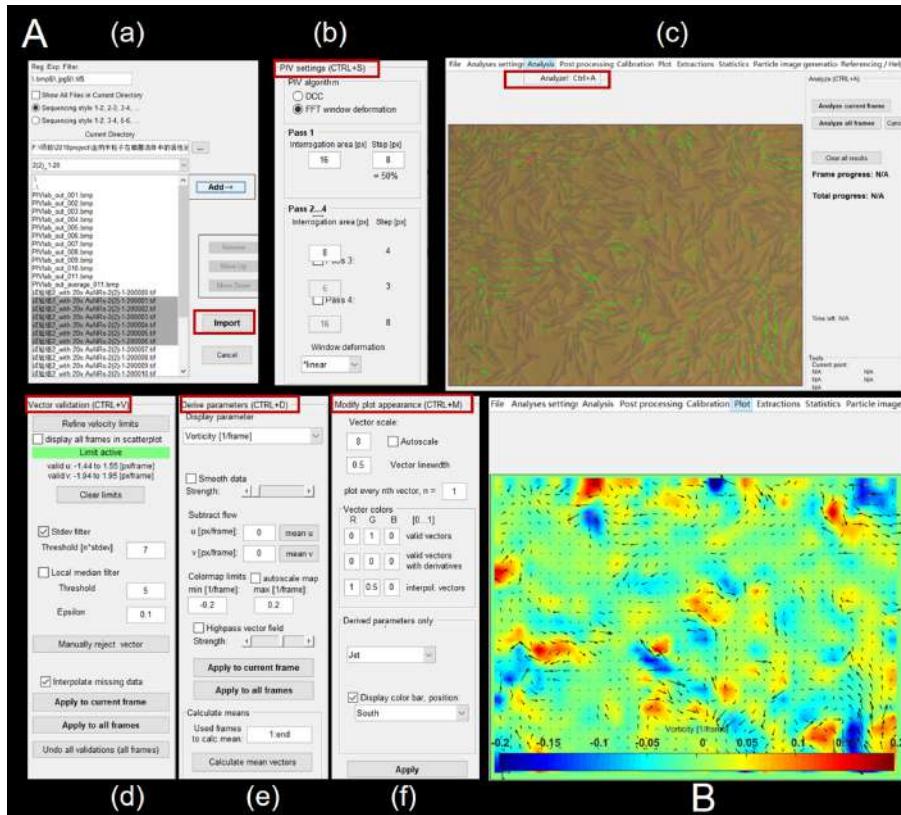


Figure 3. PIV analysis of the flow field of collective swarming bacteria. A. The general work flow while performing PIV analysis. (a) Import the image. (b) Set the PIV interrogation areas and number of passes. (c) Perform the PIV analysis of successive frames. (d) Manually refine the vector limits in the scatterplot of vector field. (e) Extract the parameters on demand. (f) Modify the plot appearance. B. A typical image of the vorticity field overlaid by the velocity vector needles.

B. Particle tracking

1. Single particle tracking by ImageJ (Figure 4A)

Load image sequences by File > import > image sequences. Open the particle tracking plugin by ImageJ > Plugins > Particle Trackers Classic > Particle tracker. Set the particle detection parameters including the particle radius, cutoff, link range and the displacement Figure 4B (a), etc. Visualize and select trajectories Figure 4B (b). Save the tables and images Figure 4B (c-d).

Note: while using ImageJ, only some of the tracers could be tracked easily due to the interference of the cells in the background.

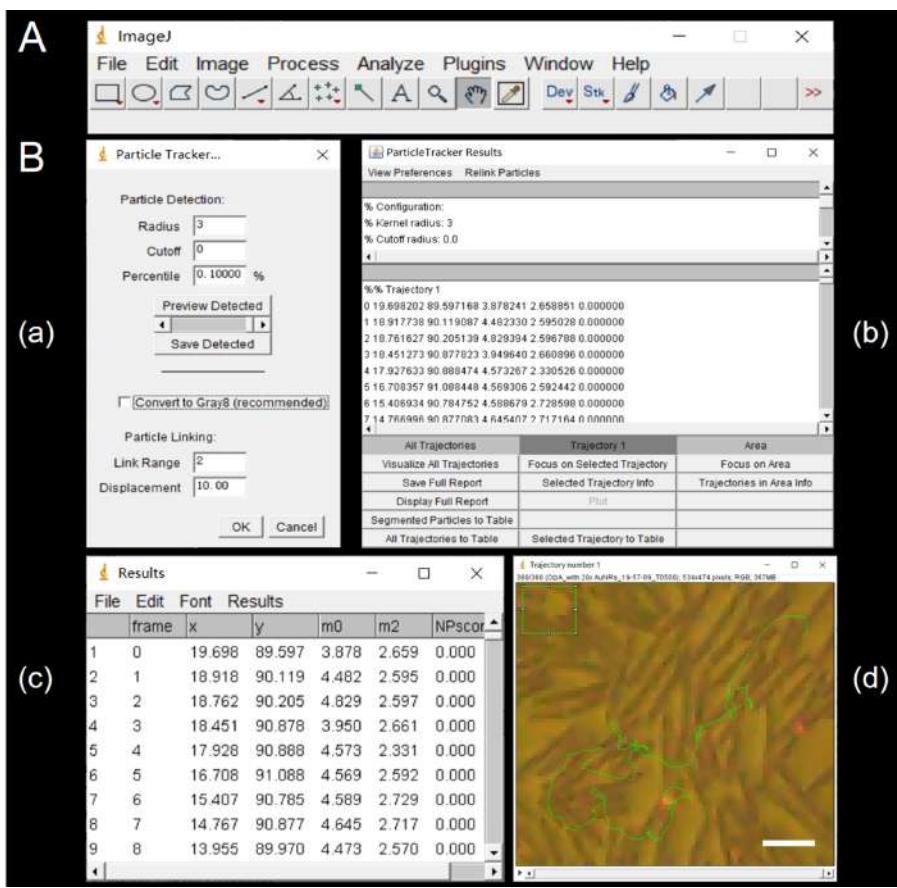


Figure 4. Single particle tracking analysis. A. The work panel of ImageJ. B. Figure (a)-(b) exhibit the work flow when using “Particle tracker” plugins. (c) The results show the coordinates of AuNRs in each frame. (d) The panel shows the overlay of the AuNRs trajectory on the original image. The scale bar is 5 μm .

2. Multi-particle tracking

To obtain the trajectories of multiple particles in successive frames, we developed a software in MATLAB. The main procedures include image preprocessing, particle recognition based on the local intensity threshold and inter-frame tracking. The codes and detailed instructions are shared in GitHub as indicated in Software.

Note: In the multi-particle tracking software, a local dynamic intensity threshold instead of a global one was set to recognize the particles from the background of cells.

C. Diffusion analysis of tracers’ trajectories

1. Mean squared displacements (MSDs)

Compute the mean squared displacement $\langle \Delta r^2(\tau) \rangle$ of each trajectory over different lag times τ . Then compute the ensemble averaged MSDs for multiple trajectories in the field of view. Plot in log-log scale. See also in the Figure 3C of original paper.

2. The distributions of the direction of motions and turning angles

Make the right side of the x-axis as positive direction arbitrarily. Define the direction of motions

as the direction of each step relative to the x-axis and the turning angles as the directions between successive steps. Plot the probability distribution function (PDF) in the semi-log scale in the Origin employing the calculated data of all trajectories in the field of view. See also the Figure 3A and 3B of original paper.

3. PDF of normalized displacements

Compute the displacements Δx over different lag times Δt . While comparing the PDF of displacements of different Δt , Δx should be normalized by $\sqrt{2D\Delta t}$ where D is the diffusion coefficient calculated from the fitting of MSD curves. Plot the PDF in semi-log scale. See also the Figure 3D of original paper.

4. Correlation analysis of angular changes and speed of tracer particles

Calculate the instantaneous velocity of a particle over time. Perform the correlation analysis of the tracers' time-dependent angular change (turning angles) and velocity in the SPSS utilizing Pearson correlation function. The p-value are less than 0.05 with the confidence level of 95%.

See also the Figures 3E and 3F of original paper.

5. Calculation of local MSD exponents with the slicing window method

Slice the trajectories into different lengths according to the chosen window size. Then calculate the MSD exponents of each trajectory segment. See the boxplot in Figure 3H of original paper.

D. Fitting of the Lévy walk model

Segment the long trajectory into “flights” which consists of several steps by “Angle method” modified from Turchin (1998). Next fit the PDF of “flights” to the pow-law function, $P(x) = C x^{-\mu}, x \geq x_{min}$ to get the exponent μ through maximum likelihood estimation method. Then justify the power-law model over the exponential model by Akaike weights (Edwards *et al.*, 2007). The detailed analysis and explanations could be found in Figure 4 and transparent methods—“6. Lévy walk models and the power law fitting” of original paper (Feng *et al.*, 2019).

Recipes

a) Soft LB agar for swarming

10 g/L Tryptone, Bacto

5 g/L Yeast extract, LD, Bacto

5 g/L NaCl

0.5% Bacto Agar

b) LB broth, solution

10 g/L Tryptone, Bacto

5 g/L Yeast extract, LD, Bacto

5 g/L NaCl

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Competing interests

The authors have no competing interests.

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A Mismatch-tolerant RT-LAMP Method for Molecular Diagnosis of Highly Variable Viruses

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[Abstract] Loop-mediated isothermal amplification (LAMP) has been widely used in the detection of pathogens. However, there are usually numerous variants in one viral pathogen and primers employed in LAMP can hardly match all these variants. The mismatches between the primers and the viral genomes, especially those at the 3'-end of the primers, hinder LAMP reactions, leading to failure of the detection. Here, we present a mismatch-tolerant RT-LAMP protocol, which utilizes the 3'-5' exonuclease activity of the Q5 high-fidelity DNA polymerase to remove potential mismatched bases at the 3'-end of the primers during LAMP amplification. Using HIV-1 as a proof-of-principle, we showed that this protocol could represent a promising tool for accurate detection of genetically unstable viruses in laboratory, hospital and field.

Keywords: Mismatch-tolerant RT-LAMP, Highly variable viruses, Mutant, HIV-1, Visual detection, High-fidelity DNA polymerase

[Background] Emerging and re-emerging infectious diseases are serious threats to global public health (Mehand *et al.*, 2018). Many outbreaks and epidemics have been caused by viruses, such as HIV-1, HCV, MERS-CoV, Ebola virus, A/H7N9 influenza virus, and more recently Zika virus. These viral diseases have led to high morbidity and mortality that disproportionately impacted on low-income countries (Van Doorn, 2017; Fenollar and Mediannikov, 2018; Waldman and Balskus, 2018). Rapid and accurate diagnosis of viral pathogens is crucial for the prevention and control of viral infectious diseases (Mehand *et al.*, 2018).

Isothermal amplification techniques represent a promising direction for the development of point-of-care testing (POCT) diagnostic tools especially in the low-income countries or resource-limited settings (de Paz *et al.*, 2014). Loop-mediated isothermal amplification (LAMP) is the most widely used isothermal amplification technology in biomedical research (Notomi *et al.*, 2000). Its principle is auto-cycling strand displacement DNA amplification reaction using Bst DNA polymerase with high strand displacement activity under isothermal condition. LAMP generally uses three pairs of primers, and two inner primers (FIP and BIP) are responsible for initiating the self-primed DNA synthesis of the dumbbell form DNA. However, the biggest challenge for the detection of viruses using LAMP is the high genetic diversity of some viral genomes, which exist in the forms of genotypes, subtypes, and/or quasispecies (Sanjuan *et*

al., 2010; Domingo and Perales, 2018). These diverse forms can easily cause mismatches with primers during their amplification, thereby resulting in a low sensitivity of detection and a limited spectrum of detection (Zhang *et al.*, 2017; Li *et al.*, 2019; Zhou *et al.*, 2019). It is virtually impossible to detect all variants or serotypes in one LAMP assay as the conserved regions in the genomes are usually too short to completely match the long (approximately 40 nt) inner primers (FIP and BIP) of the assay. Therefore, an underestimate of viral load or even failure of detection is common using RT-LAMP method, especially for highly variable RNA viruses (Waldman and Balskus, 2018; Zhou *et al.*, 2019). This may be the most important reason that limits the commercial application of LAMP in the diagnosis of viral infectious diseases (Wong *et al.*, 2018). To overcome this problem, we recently developed a mismatch-tolerant RT-LAMP method that contains a minuscule amount of high-fidelity DNA polymerase and utilizes its 3'-5' exonuclease activity to remove potential mismatched bases at the 3'-end of the primers during amplification (Zhou *et al.*, 2019). The new method was demonstrated to be especially suited for the detection of highly variable viruses (Zhou *et al.*, 2019). In this paper, we provide a detailed protocol of the mismatch-tolerant RT-LAMP method using HIV-1 detection as an example (Table 1).

Table 1. Primers used for the HIV-1 RT-LAMP assay

Types	Primer	Sequence (5'-3')	Sources
Outer primers	AceIN-F3	CCMMTTGGAAAGGACCAGC	This work
	AceIN-B3b	AACATACATATGRTGYTTACTA	
	AceIN-B3a	TCTTGAAAYATACATATGRTG	
	AceIN-FIPf	CTTGGCACTACYTTATGTCACTAAARCTYCTCTGGAAAGGTG	
Inner primers	AceIN-FIPe	CTTGGTACTACYTTATGTCACTAAARCTACTCTGGAAAGGTG	(Owieja <i>et al.</i> , 2015)
	AceIN-BIP	GGAYATGGAAAACAGATGGCAGCCATGTTCTAACYTCATCCTG	
Loop primers	AceIN-LF	TCTTGATTACTACTGCCCTT	This work
	AceIN-LB	GTGMTGATTGTGTGGCARGTAG	

Materials and Reagents

- 117.Axygen® MicroVolume Extended-Length Filtered Pipet Tips (Axygen, catalog number: TXLF10)
- 118.Axygen® Universal Fit 100 µl Filtered Pipet Tips (Axygen, catalog number: TF100RS)
- 119.Axygen® Universal Fit 200 µl Filtered Pipet Tips (Axygen, catalog number: TF200RS)
- 120.Axygen® Universal Fit 1,000 µl Filtered Pipet Tips (Axygen, catalog number: TF1000LRS)
- 121.Axygen 1.5 ml Snaplock Microtubes (Axygen, catalog number: MCT150CS)
- 122.Axygen 0.2 ml PCR® Tubes (Axygen, catalog number: PCR02C)
- 123.LightCycler® 480 Multiwell Plate 96,white (Roche, catalog number: 4729692001)
- 124.LightCycler® 8-Tube Strips, white (Roche, catalog number: 6612601001)
- 125.DreamTaq™ Green PCR Master Mix (2x) (Thermo Fisher Scientific, catalog number: k1081)
- 126.pUC57-IN (containing partial fragment of HIV-1 integrase gene: AF033819.3. the sequence is 5'-ACGGTTAGGGCCGCCTGTTGGTGGCGGGAAATCAAGCAGGAATTGGAATTCCC

TACAATCCCCAAAGTCAAGGAGTAGTAGAATCTATGAATAAAGAATTAAAGAAAATTATAGGA
CAGGTAAGAGATCAGGCTAACATCTAACAGACAGCAGTACAAATGGCAGTATTCCACAA
ATTTAAAAGAAAAGGGGGATTGGGGGTACAGTCAGGGAAAGAATAGTAGACATAA
TAGCAACAGACATACAAACTAAAGAATTACAAAACAAATTACAAAATTCAAATTTGGG
TTTATTACAGGGACAGCAGAAATCCACTTGAAAGGACCAGCAAAGCTCCTCTGGAAAG
GTGAAGGGCAGTAGTAATACAAGATAATAGTGACATAAAGTAGTGCCAAGAAGAAAAGC
AAAGATCATTAGGGATTATGGAAAACAGATGGCAGGTGATGATTGTGGCAAGTAGACAG
GATGAGGATTAGAACATGGAAAAGTTAGTAAACACCATATGTATGTTCAGGGAAAGCTA
GGGGATGGTTTATAGACATCACTATGAAAGCCCTATCGGATCCCAGGGCCGTCGACTG-3')
(Synthesized by Shanghai BioSune Biotechnology Co., Ltd.)

- 127.Qubit™ RNA HS Assay Kit RNA (Life Technologies, catalog number: Q32855)
- 128.WarmStart RTx Reverse Transcriptase (NEB, catalog number: M0380L)
- 129.Q5® High-Fidelity DNA Polymerase (NEB, catalog number: M0491L)
- 130.*Bst* 2.0 DNA Polymerase (NEB, catalog number: M0537L)
- 131.Fast Mutagenesis System (Transgen, catalog number: FM111)
- 132.QIAgen Viral RNA Mini Kit (Qiagen, catalog number: 52906)
- 133.QIAquick® Gel Extraction Kit (Qiagen, catalog number: GC-28706)
- 134.HiScribe T7 High Yield RNA Synthesis Kit (NEB, catalog number: E2040S)
- 135.WarmStart® Colorimetric LAMP 2x Master Mix (DNA & RNA) (with cresol red) (NEB, catalog number: M1800S)
- 136.Isothermal Amplification Buffer Pack (NEB, catalog number: B0537S)
- 137.Magnesium Sulfate ($MgSO_4$) Solution (NEB, catalog number: B1003S)
- 138.dNTP Set, 100 mM Solutions (Thermo Fisher, catalog number: R0186)
- 139.SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, catalog number: S34854)
- 140.Nuclease-Free Water (not DEPC-Treated) (Ambion™, catalog number: AM9938)
- 141.Agarose (Biowest, catalog number: BY-R0100)
- 142.Ultra-pure water (Genview, catalog number: GU3313-500)
- 143.GelRed Nucleic Acid Straining Dye (10000x) (TOROIVD, catalog number: RSD100-25)
- 144.Plasma samples (Previous samples from our laboratory)
- 145.Yeast extract (Oxoid, catalog number: LP0021)
- 146.Tryptone (Oxoid, catalog number: LP0042)
- 147.Agar (Shangxiang, catalog number: 120420)
- 148.Sodium chloride (HuShi, catalog number: 10019318)
- 149.Ampicillin trihydrate (Solarbio, catalog number: A7490-5)
- 150.50x TAE buffer (Meilunbio, catalog number: MA0004)
- 151.2000 DNA Marker (Yeasen, catalog number: 10501ES60)
- 152.5000 DNA Marker (Yeasen, catalog number: 10504ES60)
- 153.TIANprep Mini Plasmid Kit (Tiangn, catalog number: DP103-03)
- 154.Liquid LB medium (see Recipes)

- 155. 100 mg/ml ampicillin solution (see Recipes)
- 156. Ampicillin-resistant solid medium (see Recipes)
- 157. Ampicillin-resistant liquid LB medium (see Recipes)
- 158. 2% agarose gel (see Recipes)
- 159. 1% agarose gel (see Recipes)

Equipment

- 32. 0.5-10 μ l Eppendorf Research[®] plus Adjustable Volume Pipettes (Eppendorf, catalog number: I32693E)
- 33. 10-100 μ l Eppendorf Research[®] plus Adjustable Volume Pipettes (Eppendorf, catalog number: 251596Z)
- 34. 20-200 μ l Eppendorf Research[®] plus Adjustable Volume Pipettes (Eppendorf, catalog number: 4830359)
- 35. 100-1,000 μ l Eppendorf Research[®] plus Adjustable Volume Pipettes (Eppendorf, catalog number: 4847859)
- 36. NanoDrop[™] 2000 Spectrophotometer (Thermo Fisher, model: NanoDrop[™] 2000, catalog number: ND-2000)
- 37. LightCycler[®] 96 System (Roche, catalog number: 05815916001)
- 38. Bio-Rad CFX96[™] Real-Time PCR System (Bio-Rad, catalog number: 785BR18555)
- 39. Eppendorf Centrifuge 5417R (Eppendorf, model: 5417R)
- 40. Eppendorf Mastercycler nexus (Eppendorf, catalog number: 6325ZK904949)
- 41. Tanon Gel Image System (Tanon, model: 2500)
- 42. Tanon EPS 300 (Tanon, model: 300)
- 43. Tanon electrophoresis tank (Tanon, model: 400)

Software

- 9. LightCycler[®] 96 System software (Roche)
- 10. Bio-Rad CFX Manager 3.1 software (Bio-Rad)

Procedure

M. Preparation of HIV-1 wild type and mutant RNA standards

1. Construct two mutant pUC57-IN plasmids using the fast mutagenesis system according to the following steps. Primers for construction of the mutant plasmids are in Table 2.
 - a. Prepare the PCR reaction mixes (Table 3), PCR cycling condition: Enzyme activation and pre-denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s and extension at 72 °C for 1 min, followed by extending at 72 °C for 10

min.

Table 2. Primers for construction of two HIV-1 mutant plasmids

Primer	Sequence (5'-3')
AceIN-F3 Mu1-G F	CTTGAAAGGACCAG G AAAGCTCCTC
AceIN-F3 Mu1-G R	C CTGGTCCTTCAAAGTGGATTCTG
AceIN-F3 Mu2-A F	CTTGAAAGGACCAG A AAAGCTCCTC
AceIN-F3 Mu2-A R	T CTGGTCCTTCAAAGTGGATTCTG

Note: Introduced mutant is shown in Red font. F: forward primer; R: reverse primer; Mu: mutant.

Table 3. PCR reaction mixes of the construction of two HIV-1 mutants

Component	Volume (μ l)
pUC57-IN (1-10 ng)	2
Mu F (10 μ M)	1
Mu R (10 μ M)	1
2×TransStart FastPfu PCR Supermix	25
Nuclease-free water	21
total	50

Note: F: forward primer; R: reverse primer; Mu: mutant.

- b. Electrophoresis detection: measure 10 μ l PCR product using 1% agarose gel electrophoresis at a constant voltage (140 V) for about 30 min.
 - c. PCR product digestion: add 1 μ l of DMT enzyme to the remaining PCR product, mix and incubate for 1 h at 37 °C.
 - d. Add 5 μ l of DMT digestion product to 50 μ l of competent cells, mix and place on ice for 30 min. Then place the mixture at 42 °C for 45 s, and on ice for 2 min.
 - e. Add 250 μ l of room temperature LB medium (without antibiotics) to the mixture, 200 rpm, 37 °C for 1 h.
 - f. Spread 100 μ l of the bacterial solution evenly on a plate containing 100 μ g/ml ampicillin and incubate overnight in a 37 °C incubator.
 - g. Pick the mono-clones into 3 ml LB medium containing 100 μ g/ml ampicillin, for shaking culture (200 rpm), at 37 °C for 10-12 h.
 - h. Extract the plasmid DNA for Sanger sequencing (Shanghai Platinum Company) and verify the presence of the mutation. Store the mutated plasmids at -20 °C for subsequent experiments.
2. Amplify the HIV-1 integrase segment using T7 promotor-containing primer pair (HIV T7-F: TAATACGACTCACTATAGACGGTTAGGGCCGCCTGT and HIV R: CAGTCGACGGGCCGGGA) with the wild-type and mutant HIV-1 plasmids as templates.
 - a. In a 0.2 ml PCR® tube with cap, a PCR reaction mix includes 25 μ l 2x DreamTaq™ Green

PCR Master Mix, 2 μ l of 10 μ M of each primer (HIV T7-F and HIV R primer) and 2 μ l HIV-1 plasmid in a final volume of 50 μ l.

- b. The cycling condition is enzyme activation and pre-denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, fully extending at 72 °C for 10 min.
3. Measure the PCR product using 2% agarose gel electrophoresis at a constant voltage (140 V) for about 30 min.
4. After electrophoresis, cut the specific PCR product from the gel under UV control and purify according to the manufacturer's instruction using the QIAquick® Gel Extraction Kit.
5. Obtain RNAs through *in vitro* transcription with the purified DNA as templates using the HiScribe T7 High Yield RNA Synthesis Kit.
6. Quantify the obtained RNA using NanoDropTM 2000 Spectrophotometer, and calculate the RNA copy number using the following formula:

$$\text{RNA copies/ml} = [\text{RNA concentration (g/ml)} / (\text{nt transcript length} \times 340)] \times 6.022 \times 10^{23}$$

7. Dilute RNA to 10⁴ copies/ μ l, aliquot to 40 μ l in a 0.2 ml PCR® tube with cap and store at -80 °C until use.

N. Plasma sample viral RNA extraction

1. Extract viral RNA from 140 μ l of plasma using the QIAgen Viral RNA Mini Kit following the manufacturer's instruction.
2. Elute the extracted RNA in 50 μ l of nuclease-free water, aliquot to 25 μ l per tube, and store at -80 °C until use.

O. Qualitative detection of HIV-1 by the real-time mismatch-tolerant HIV-1 RT-LAMP assay

1. Preparation of mixed primer sets (Table 4)

Prepare the mixed primer set 1 by adding 5 μ l 100 μ M each of primers AceIN-F3, AceIN-B3a, and AceIN-B3b into 85 μ l nuclease-free water; the mixed primer set 2 includes 20 μ l 100 μ M each of primers AceIN-FIPf and AceIN-FIPe, 40 μ l 100 μ M AceIN-BIP, and 20 μ l nuclease-free water; the mixed primer set 3 includes 20 μ l 100 μ M each of primers AceIN-LF and AceIN-LB, and 60 μ l nuclease-free water.

2. Preparation of the real-time RT-LAMP mix (Table 5)

In a 96-well PCR plate or 8-tube PCR strip, each 25 μ l reaction mix includes 3 μ l RNA template (RNA standard or RNA extracts from clinical sample), 2.5 μ l 10x isothermal amplification buffer, 1 μ l 100mM MgSO₄, 3.5 μ l 10 mM dNTPs, 0.075 μ l 2 units of Q5 high-fidelity DNA polymerase, 1 μ l 8 units of *Bst* 2.0 DNA polymerase, 0.5 μ l 15 units of warmstart RTx reverse transcriptase, 1 μ l 1 mM SYTO 9, and 1 μ l each mixed primer set (mixed primer set 1-3 in Table 4).

Table 4. Mixed primer sets preparation of the mismatch-tolerant RT-LAMP assay

HIV-1 RT-LAMP assay		Regular LAMP assay	
Mixed primer set	Volume (μ l)	Primers	Volume (μ l)
Mixed primer set 1 (25 \times)	100 μ M AceIN-F3	5	100 μ M F3
	100 μ M AceIN-B3a	5	100 μ M B3
	100 μ M AceIN-B3b	5	Nuclease-free water
	Nuclease-free water	85	
	total	100	100
Mixed primer set 2 (25 \times)	100 μ M AceIN-FIPf	20	100 μ M FIP
	100 μ M AceIN-FIPE	20	100 μ M BIP
	100 μ M AceIN-BIP	40	Nuclease-free water
	Nuclease-free water	20	
	total	100	100
Mixed primer set 3 (25 \times)	100 μ M AceIN-LF	20	100 μ M Loop F
	100 μ M AceIN-LB	20	100 μ M Loop B
	Nuclease-free water	60	Nuclease-free water
	total	100	total
			100

Note: The regular assay is recommended when no degenerate primers is used.

- RT-LAMP cycling condition: Perform the reaction at 62 °C for 1 min by 60 cycles. Collect fluorescence signal at each cycle.

P. Colorimetric RT-LAMP detection (Table 5)

- Preparation of the colorimetric RT-LAMP mix (Table 5)

In an 8-tube PCR strip, each 25 μ l reaction mix includes 12.5 μ l warmstart colorimetric LAMP 2x master mix, 1 μ l each mixed primer set (mixed primer sets 1-3 in Table 4), 0.075 μ l 2 units of Q5 high-fidelity DNA polymerase, and 3 μ l of RNA template (RNA standard or RNA extracts from clinical sample).

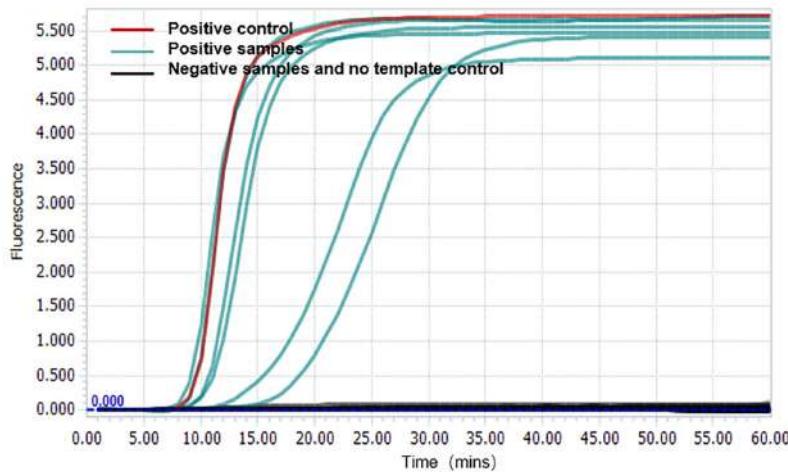
- Colorimetric RT-LAMP cycling condition: Perform the reaction at 62 °C for 50 min. Observe the color change at 20-, 30-, 40-, and 50-min time points by naked eyes.

Data analysis

- The result of the real-time mismatch-tolerant RT-LAMP assay can be seen with LightCycler® 96 system software, Bio-Rad CFX manager 3.1 software (Figure 1) or softwares implemented in other real-time PCR machines. Positive results (with the presence of HIV-1 templates) show clear "S" type amplification curves, and negative results (without HIV-1 template) have no amplification curve. The Ct values (time to appearance of the amplification curve) are negatively

related to the amount of template input.

A Light Cycler® 96 System



B Bio-Rad CFX 96™ Real-Time PCR System

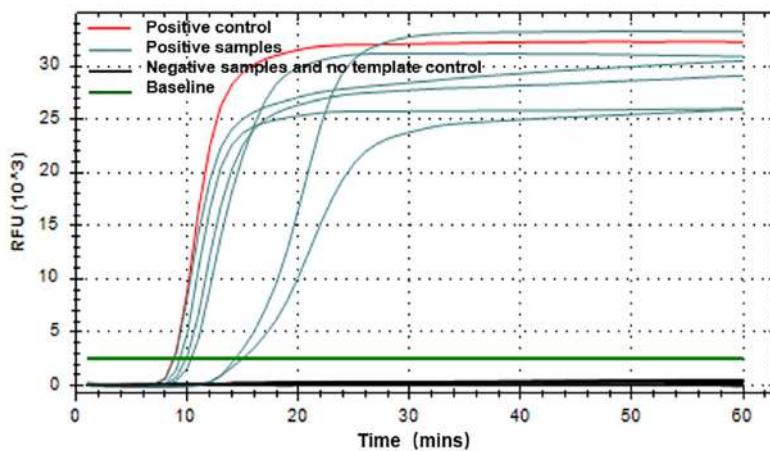


Figure 1. Amplification curves of the real-time mismatch-tolerant HIV-1 RT-LAMP. Amplification curves using LightCycler® 96 system (A) or Bio-Rad CFX96 TM Real-Time PCR system (B). HIV-1 RNA standard used as a positive control is shown in red, clinical samples from HIV-positive subjects are shown in cyan, and samples from HIV-negative subjects or that do not have template are shown in black.

2. In the Colorimetric RT-LAMP detection assay, the results can be judged by naked eyes. A clear color change from burgundy to orange or yellow is considered as HIV-1 positive at 30-50 min which is dependent on the set cut-off (Figure 2).

Table 5. Real-time and colorimetric reaction mixes of the mismatch-tolerant RT-LAMP assay

Component	Real-time RT-LAMP (μl)	Colorimetric RT-LAMP (μl)
10× Isothermal Amplification Buffer	2.5	-
WarmStart Colorimetric LAMP 2x Master Mix	-	12.5
MgSO ₄ (100 mM)	1	-
dNTPs (10 mM)	3.5	-
<i>Bst</i> 2.0 DNA polymerase (8 U)	1	-
Warmstart RTx reverse transcriptase (15 U)	0.5	-
Q5 high-fidelity DNA polymerase (0.2 U/μl)	0.75	0.75
SYTO 9 (1×)	1	-
Mixed primer set 1 (25×)	1	1
Mixed primer set 2 (25×)	1	1
Mixed primer set 3 (25×)	1	1
RNA template	3	3
Nuclease-free water	8.75	5.75
Total	25	25

Note: Obtain 0.2 U/μl Q5 high-fidelity DNA polymerase by ten-fold dilution of 2 U/μl Q5 enzyme stock.

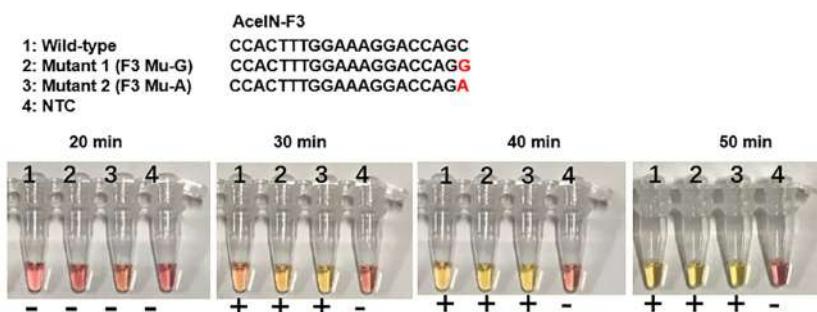


Figure 2. Colorimetric RT-LAMP detection of HIV-1. The color change from burgundy to orange or yellow is considered as positive (+). NTC: no template control. The mutated base is marked in red. AcIN-F3 is an outer forward primer.

Notes

1. The LAMP reaction is very sensitive. Attention should be paid to avoid contamination during the operation, and a stringent laboratory compartmentalization is strongly recommended for LAMP and other amplification assays.
2. To avoid potential contamination, agarose gel electrophoresis of LAMP products is not

encouraged.

3. Because only a small amount of Q5 high-fidelity DNA polymerase is used in each reaction, the enzyme stock can be diluted to a lower concentration (e.g., 0.2 U/μl) to reduce pipetting error.
4. Other high-fidelity DNA polymerases can also be used instead of Q5 enzyme in the reaction. The recommended optimal concentration of high-fidelity DNA polymerase is between 0.1 and 0.3 units per 25 μl LAMP reaction.
5. The exact amount of Q5 high-fidelity DNA polymerase per 25 μl LAMP reaction needs to be optimized for detection of each specific virus.
6. If there are no degenerate primers to be used, the primer mix can be prepared based on the standard assay (Table 2).
7. The amount of RNA template per 25 μl LAMP reaction can be adjusted between the range of 0-11.75 μl for the real-time version, and 0-8.75 μl for the colorimetric version (Table 3).

Recipes

1. Liquid LB medium
10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride in 1 L of ultra-pure water
Mix and autoclave it
2. 100 mg/ml ampicillin solution
1 g of ampicillin powder in 5 ml of ultra-pure water
After it was dissolved, add ultra-pure water to make it to 10 ml
Dispense the 100 mg/ml ampicillin solution into a 1.5 ml EP tube and store at -20 °C until use
3. Ampicillin-resistant solid medium
10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride and 15 g of agar in 1 L of ultra-pure water
Mix and autoclave it
Add 1 ml of 100 mg/ml ampicillin solution and mix it
Pour about 10 ml on each plate, and store at 4 °C after solidification
4. Ampicillin-resistant liquid LB medium
Add 50 μl of 100 mg/ml ampicillin solution in 50 ml liquid LB medium and mix it
5. 2% agarose gel
2 g of agarose in 100 ml of 1x TAE and 10 μl 10000x GelRed
6. 1% agarose gel
1 g of agarose in 100 ml of 1x TAE and 10 μl 10000x GelRed

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Competing interests

The authors declare no competing interests.

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I Plate-based Assay for Studying How Fungal Volatile Compounds (VCs) Affect Plant Growth and Development and the Identification of VCs via SPME-GC-MS

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[Abstract] Biogenic volatile compounds (VCs) mediate various types of crucial intra- and inter-species interactions in plants, animals, and microorganisms owing to their ability to travel through air, liquid, and porous soils. To study how VCs produced by *Verticillium dahliae*, a soilborne fungal pathogen, affect plant growth and development, we slightly modified a method previously used to study the effect of bacterial VCs on plant growth. The method involves culturing microbial cells and plants in I plate to allow only VC-mediated interaction. The modified protocol is simple to set up and produces reproducible results, facilitating studies on this poorly explored form of plant-fungal interactions. We also optimized conditions for extracting and identifying fungal VCs using solid phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS).

Keywords: *Arabidopsis thaliana*, GC-MS, I plate, Plant-fungal interaction, SPME, *Verticillium dahliae*, Volatile compounds

[Background] Volatile compounds (VCs) have been shown or suggested to play varied and crucial roles in mediating organismal interactions within and across kingdoms. Plants rely on VCs to attract pollinators, seed dispersers, and parasitoids (Baldwin, 2010; Herrmann, 2010). Animals have evolved sophisticated olfactory systems to detect and respond to foods, threats, and mates through volatile cues (Buck, 2004). Similarly, microbial VCs seem to perform diverse functions such as suppressing competitors, regulating their population density, and controlling morphological transitions (Bailly and Weisskopf, 2012; Bennett et al., 2012; Bitas et al., 2013). Roles of microbial VCs in plant growth, development, and stress response have been investigated using several experimental setups that physically separate microbial cells from plants so that only VC-mediated interaction can occur (Ryu et al., 2003; Kai and Piechulla, 2009; Xie et al., 2009; Hung et al., 2013; Vaishnav et al., 2015). Among them, a method employing bipartite Petri plate, also called I plate, has been most frequently used. We adopted I plate to study the effect of VCs produced by soilborne fungal pathogens on plant growth, development, and responses to biotic and abiotic stresses (Bitas et al., 2015; Li and Kang, 2018; Li et al., 2018b). In addition, we optimized a scheme for VC extraction and analysis to help identify fungal VCs that are responsible for modulating plant growth and development (Li et al., 2018b).

Here, we provide a detailed protocol for setting up an I plate assay used for evaluating the effect of

VCs produced by *Verticillium dahliae*, a devastating soilborne fungal pathogen that infects hundreds of plant species, on *Arabidopsis thaliana*. This protocol enables rapid and straightforward determination of if and how fungal VCs affect plants. We also describe a protocol for capturing VCs through solid phase microextraction (SPME) and analyzing extracted VCs via gas chromatography-mass spectrometry (GC-MS). In combination, these protocols will help explore how VCs produced by diverse fungi affect plants and can also be applied to study VC-mediated interactions between microbes.

Materials and Reagents

1. Surgical blades #10 and #11
2. Parafilm (Bermis, catalog number: PM-99)
3. Paper towel
4. 10 µl and 1,000 µl micropipette tips
5. 100 x 15 mm I plate (VWR, catalog number: 25384-310)
6. 100 x 100 mm square plate (VWR, catalog number: 10799-140)
7. 100 x 15 mm (VWR, catalog number: 25384-302) and 60 x 15 mm (VWR, catalog number: 25384-092) Petri plates
8. 1.7 ml microcentrifuge tube (VWR, catalog number: 87003-294)
9. 25 ml serological pipette (VWR, catalog number: 89130-900)
10. Filter unit with 0.2 µM cellulose membrane (Nalgene, catalog number: 121-0020)
11. 1.5 ml sample vial (Shimadzu, catalog number: 221-34274-91), white cap with septum
12. *V. dahliae* strains PD322 and PD413 (conidial suspension in 20% glycerol and stored at -80 °C)
13. *A. thaliana* ecotype Col-0 seeds (Lehle Seed Co.)
14. Sterile MilliQ water
15. Murashige and Skoog (MS) basal medium (Sigma-Aldrich, catalog number: M0404-10L)
16. Sucrose (Alfa Aesar, catalog number: A15583)
17. Granulated agar (Difco, catalog number: 214530)
18. Potato dextrose agar (PDA) (Difco, catalog number: 213400)
19. 200 proof ethanol (KOPTEC, catalog number: 64-17-5)
20. 6% sodium hypochlorite (CLOROX)
21. n-Hexane (EMSURE, catalog number: 1043744000)
22. C₇-C₃₀ saturated alkanes (Sigma-Aldrich, catalog number: 49451-U)
23. 99.99% pure helium gas
24. 0.5x PDA medium (see Recipes)
25. MS agar medium (see Recipes)

Equipment

1. Pyrex glass bottle

2. 10 μ l and 1,000 μ l micropipettes
3. Scalpel
4. Forceps
5. Cork borer (5 mm in diameter)
6. SPME fiber holder (Supelco, catalog number: 57330-U)
7. 15 ml clear glass vial (Supelco, catalog number: 27159), screw cap with PTFE/silicone septum
8. SPME fiber assembly with 50/30 μ m DVB/CAR/PDMS fiber coating (Supelco, catalog number: 57328-U)
9. Electric pipette controller (Drummond Scientific Co., catalog number: 4-000-110-TC)
10. Vortex (VWR, model: Genie 2)
11. Table-top centrifuge (Eppendorf, model: 5417C)
12. Table-top shaker (VWR, catalog number: 57018-754)
13. Analytical balance (Mettler Toledo, model: AE-100)
14. Dissecting microscope (Zeiss, model: Stemi 2000-C)
15. Incubator (Sheldon Manufacturing, model: 1510E)
16. Plant growth chamber (Conviron, model: CMP5090)
17. Flexible-arm electrode holder (Mettler Toledo, catalog number: 30266628)
18. GC-MS system (Shimadzu, model: GCMS-QP2010 ultra) equipped with AOC-20i auto injector (Shimadzu, catalog number: 221-72315-48)
19. Rtx-Wax capillary (60 m, 0.25 mm ID and 0.25 μ m d_f) column (Restek, catalog number: 12426)
20. 4 °C refrigerator
21. Autoclave

Software

1. ImageJ (version 1.52a)
2. GC-MS Solution (Shimadzu, version 2.72), a package supporting GC-MS real-time and post-run analyses
3. National Institute of Standards and Technology (NIST) Mass spectral library (Shimadzu, version 11)

Procedure

A. I plate assay (Figure 1)

1. Use a sterile 10 μ l pipette tip to streak *V. dahliae* stock on 0.5x PDA (Recipe 1) plate in a zigzag pattern and incubate at 22 °C for 10 days.
2. Surface sterilize *A. thaliana* seeds as follows:
 - a. Add 1 ml 95% ethanol to a 1.7 ml microcentrifuge tube containing seeds, vortex, and incubate for 1 min.

- b. After removing ethanol, wash once with sterile MilliQ water and discard the water.
- c. Add 1 ml 6% sodium hypochlorite solution, vortex, and incubate for 15 min by shaking at 100 rpm.
- d. Wash twice with sterile MilliQ water after removing the sodium hypochlorite solution.
- e. Incubate seeds in 1 ml sterile MilliQ water at 4 °C in darkness for 3 days.
3. Prepare square plates with MS agar (Recipe 2) and slice the medium into 10 x 10 mm pieces using a sterilized scalpel with blade #10.
4. Hold one seed at the tip of a 10 µl micropipette using suction and then release the seed onto each agar piece. Seal the plate with two layers of Parafilm and place in a plant growth chamber set at 22 °C, 12 h light (4,500 lux, 60 µmol photons m⁻² s⁻¹), and 60% relative humidity for 7 days.
5. Prepare I plate by adding 8 ml MS agar to one compartment and 8 ml 0.5x PDA to the other compartment.
6. Transfer five *A. thaliana* seedlings (similar in size and growth stage) along with attached agar piece to the MS side of I plate using a sterilized scalpel with blade #10.
7. Use a heat-sterilized cork borer to generate culture plugs along the actively growing margin of *V. dahliae* culture and place one plug (upside down) to the far end of the PDA side of I plate using a sterilized scalpel with blade #11.
8. Seal the inoculated I plate with two layers of Parafilm and place in a plant growth chamber for a designated amount of time for each experiment (see Li et al., 2018b for specific examples).

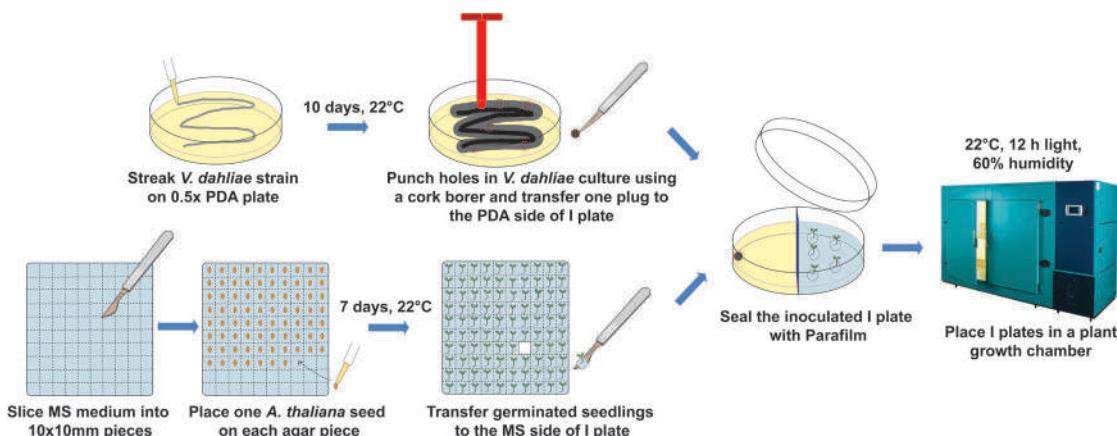


Figure 1. Workflow of the I plate-based assay

B. Extraction of VCs produced by *V. dahliae* (Figure 2)

1. Inoculate a plug of *V. dahliae* culture on 8 ml PDA slant in a 15 ml clear glass vial. Seal the vial with Parafilm.
2. Incubate at 22 °C until the culture fully covers the surface of PDA slant, which typically takes 8 days.
3. Replace Parafilm with a screw cap containing PTFE/silicone septum and incubate for one day.
4. Condition the SPME fiber before VC extraction by placing the SPME needle into the GC injection

port set at 230 °C for 1 h.

- Insert the conditioned SPME fiber in the injection port and starting the GC temperature program shown in Table 1. Desorb the fiber for 5 min in the injection port. Retract the fiber and remove the needle after 5 min and check resulting chromatograms when the program is completed. The intensity of background peaks (= blank sample) should be very low. Otherwise, repeat the desorption of the fiber.
- Extract VCs for 1 h by leaving the SPME fiber in the headspace of sampling vial. Use a flexible-arm electrode holder to lock the position of the SPME fiber holder so that the insertion depth of fiber is uniform for all extractions.

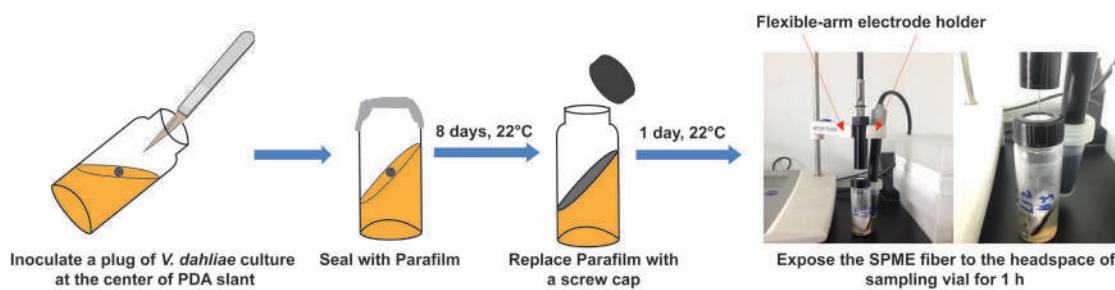


Figure 2. Workflow of volatile compound extraction

Table 1. Conditions for gas chromatography-mass spectrometry analysis

Column	Type	Rtx-Wax capillary column
	Length (m)	60
	ID (mm)	0.25
	D _f (μm)	0.25
Carrier Gas	Gas type	Helium (99.99% pure)
	Total flow	10 ml min ⁻¹
	Flow rate	1 ml min ⁻¹
	Purge flow	4 ml min ⁻¹
	Pressure	109.2 kPa
Inlet	Temperature	230 °C
	Injection mode	Splitless
Oven Temp. Program	Isothermal heating	5 min at 35 °C
	Temperature gradient	5 °C min ⁻¹ to 230 °C
	Final heating	15 min at 230 °C
Mass Spectra	Mass scan method	Total-ion-count (TIC)
	Mass scan range	35 to 500 m/z
	Run time	1.5 to 59.0 min
	Event time	0.3 s

C. GC-MS analysis of extracted VCs (Figure 3)

- Analyze the extracted VCs using a GC-MS system (manual injection mode, Figure 3A) by following the conditions described in Table 1. Insert the SPME needle into the GC injection port immediately after retracting it from each sampling vial. Desorb VCs bound to the SPME fiber for 5 min. An Rtx-Wax capillary column is used as it exhibits better retention and separation of polar VCs.
- Place the fiber in the GC injection port for 20 min for desorption before extracting another sample of VCs.
- Analyze C₇-C₃₀ saturated alkane standards using the automatic sample injection mode (Figure 3B). Connect the AOC-20i auto injector to the GC injection port. Place a 1.5 ml sample vial containing 0.5 ml solution of standards (1:50 dilution with n-hexane) on the rack of the auto injector. Inject 1 μ l solution into the GC-MS system and analyze using the same column and conditions (Table 1).

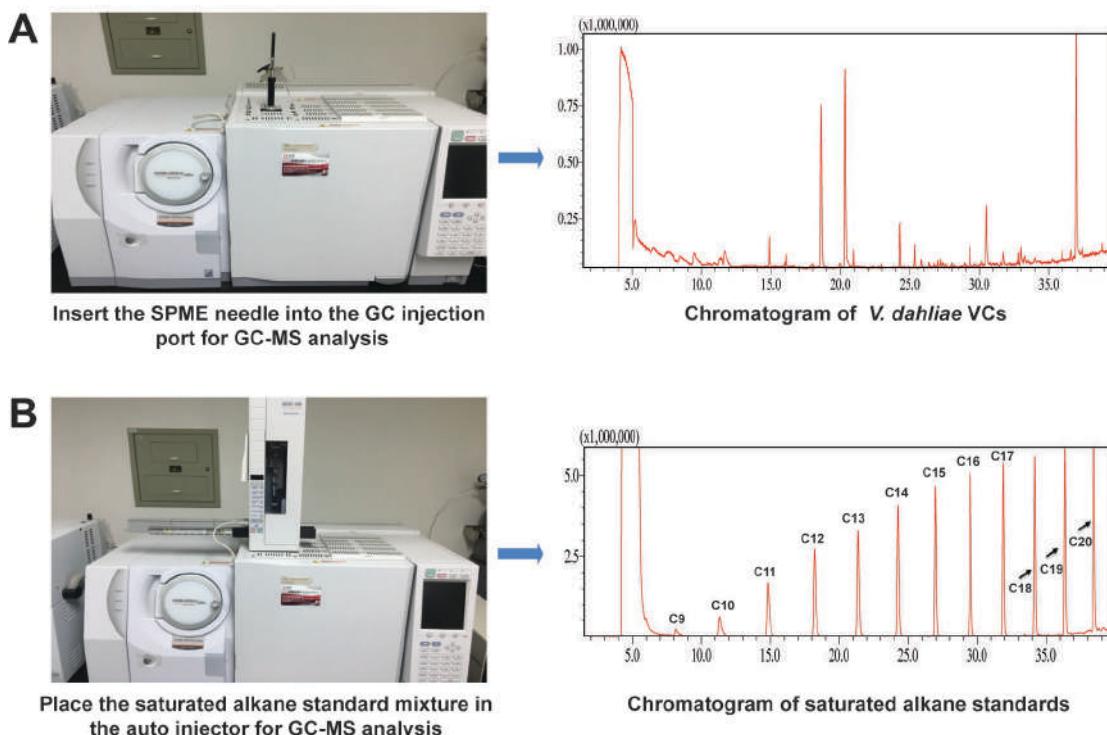


Figure 3. Gas chromatography-mass spectrometry analysis. A. Extracted volatile compounds (VCs) from *V. dahliae* were analyzed via the manual injection mode. B. Alkane standards were analyzed using the automatic sample injection mode.

Data analysis

- Assessment of root growth and development after individual VC treatments
 - After co-cultivation, use forceps to gently pull *A. thaliana* seedlings from the medium without damaging their roots.

2. Remove excess moisture on the roots using a paper towel.
3. Detaching roots from the shoot using forceps, weigh the roots immediately using an analytical balance.
4. Mount the roots on a flat surface (e.g., 100 x 15 mm Petri plate) and add 3 ml water on them.
5. Gently spread the roots using forceps so that the primary and lateral roots do not overlap.
6. Use the bottom of 60 x 15 mm Petri plate to flatten the roots (Figure 4A) for subsequent measurements:
 - a. Count the number of lateral roots, including all branches, under a dissecting microscope.
 - b. Take pictures of the roots and import them to ImageJ. Follow the instruction in ImageJ to measure the primary root length (Figure 4B).
7. Calculate the lateral root density of each sample by dividing the number of lateral roots by the primary root length.

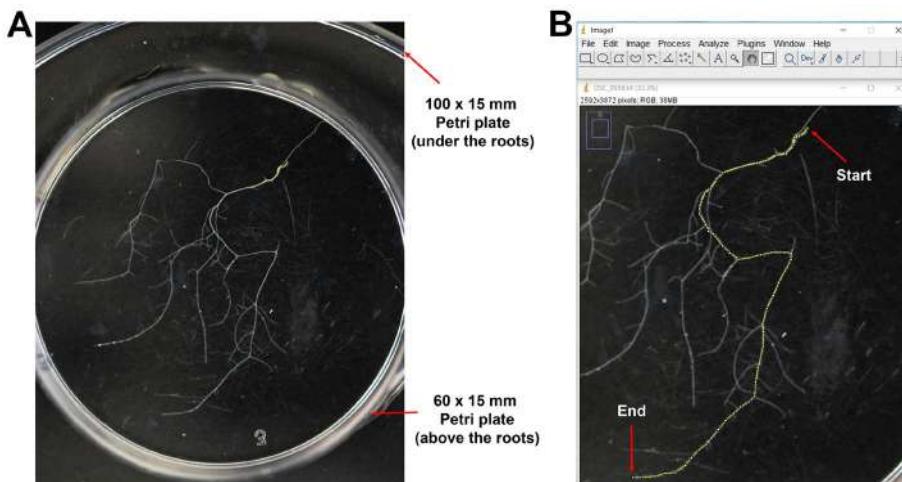


Figure 4. Analysis of *A. thaliana* roots after treating with volatile compounds produced by *V. dahliae*. A. Flattened *A. thaliana* roots by pressing with a 60 x 15 mm Petri plate. B. Snapshot of ImageJ data used to measure the length of the primary root (indicated by the yellow line) after VC treatment. The start and end of the primary root were indicated using red arrows.

B. Identification of individual compounds

1. Peak finding, peak integration, and retention time correction were performed using the post-run analysis software in the GC-MS Solution package.
2. The putative identity of each compound (peak) was determined using the following methods.
 - a. Compare resulting mass spectral profiles with reference data archived in the NIST mass spectral library (version 11). The top hit(s) with match factors $\geq 90\%$ were put on a “positive list” of tentatively identified compounds.
 - b. Calculate the retention index (RI) of each compound using the following equation (for temperature programmed chromatography) (Kováts, 1958).

$$RI_x = 100 \left[\frac{(Tx - Tn)}{(TN - Tn)} + n \right]$$

where,

x = Unknown compound in the sample

n = The number of carbons in the alkane preceding the unknown compound

N = The number of carbons in the alkane following the unknown compound

T_x = The retention time of the unknown compound

T_n = The retention time of the preceding alkane

T_N = The retention time of the following alkane

The experimentally obtained RI of each peak was compared to those in the NIST Chemistry WebBook (<https://webbook.nist.gov>, using polar columns). For positive confirmation of identity, a maximum relative deviation of $\pm 2\%$ from published values was used (Stoppacher et al., 2010).

Notes

1. The I plate assay may not be suitable for studying the effect of VCs produced by fungi that grow rapidly, as fungal mycelia may grow over the central divider of I plate and contaminate the MS medium. We removed agar strips from both sides of the divider of I plate to prevent this kind of contamination when we studied fungi that grow faster than *V. dahliae* (Bitas et al., 2015). However, for fungi like *Trichoderma*, this measure was not sufficient (Li et al., 2018a).
2. Due to the limited space of I plate, only small plants (e.g., *A. thaliana* and *Nicotiana benthamiana*) in their early growth stage are suitable for this assay.
3. Under the growth conditions used, growth promoting effect on *A. thaliana* becomes noticeable as early as after 7 days of co-cultivation with *V. dahliae* (Li et al., 2018b).
4. Because *A. thaliana* starts to initiate inflorescence development after 14 days of co-cultivation, we recommend that the duration of co-cultivation should not exceed 14 days.
5. Here, we only described how to analyze the root growth and development after VC exposure. Other traits of VC-exposed plants, such as shoot weight, chlorophyll content, physiological and molecular changes, can also be analyzed (e.g., Zhang et al., 2007 and 2008; Li et al., 2018b).
6. Several SPME fibers and GC columns may need to be evaluated to optimize the scheme for VC extraction and analysis. In our research, the DVB/CAR/PDMS 50/30 μm fiber coating extracted the largest number of VCs, and the Rtx-Wax capillary column enabled better separation of extracted VCs than the DB-5 column.
7. Analysis of VCs extracted from uninoculated PDA slant is necessary to exclude VCs derived from the medium and the environment.

Recipes

1. 0.5x PDA medium (1 L)
19.5 g PDA
Pour the autoclaved medium after cooling it down to 50 °C
2. MS agar medium (1 L)
4.5 g MS and 7.5 g agar
Add 5 ml 50% (w/v) sucrose (filter sterilized) after cooling down the autoclaved medium to 50 °C
Mix well before pouring

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Competing interests

The authors declare no conflicts of interest or competing interests.

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