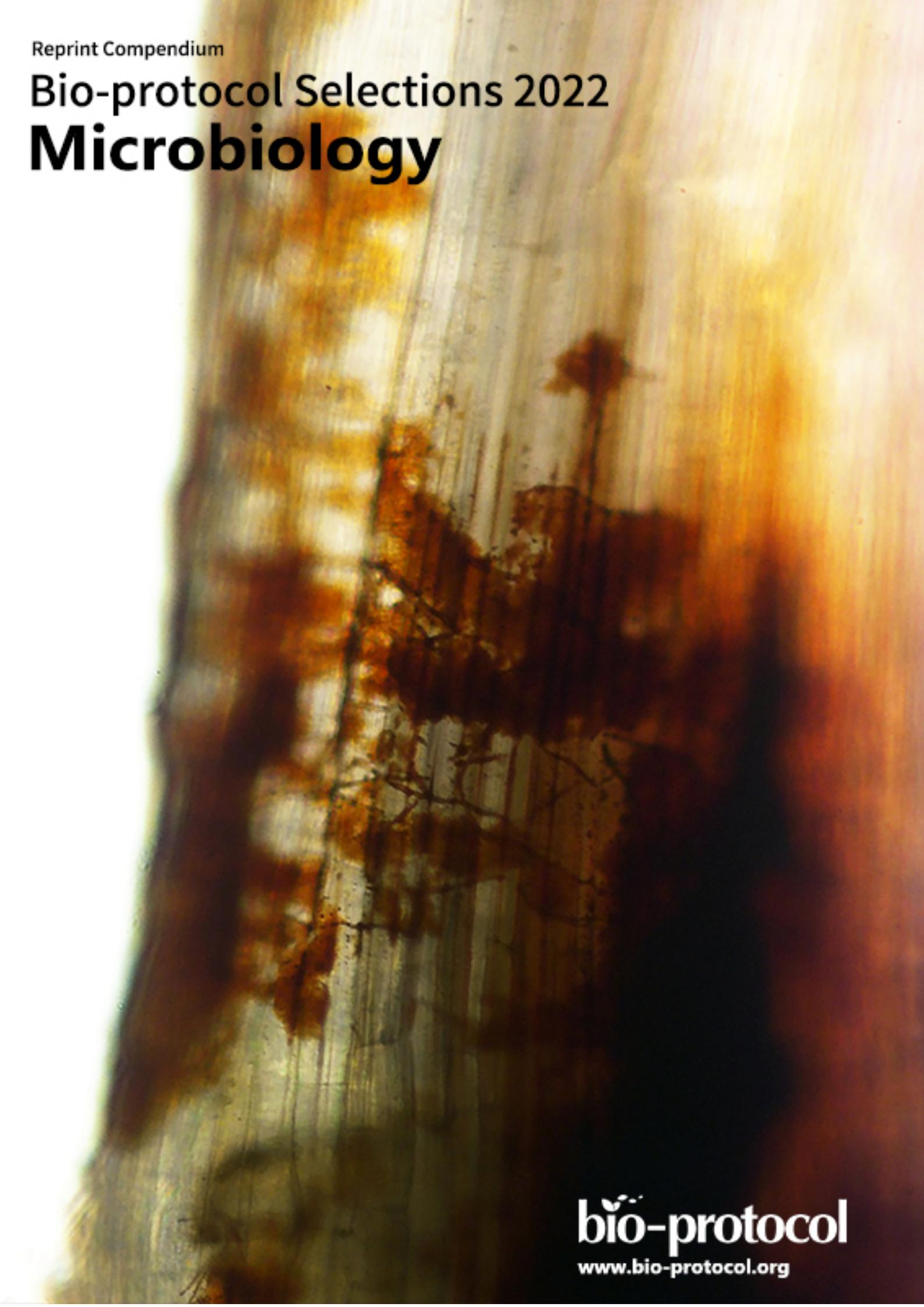


Reprint Compendium

Bio-protocol Selections 2022

Microbiology



Foreword

We are pleased to launch the 2022 *Bio-protocol* series of reprint collections, comprising some of the most used protocols published in 2021 in several research areas. This collection focuses on Microbiology.

Established in 2011 by a group of Stanford scientists, Bio-protocol aims to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. *Bio-protocol* invites contributions from authors who have published methods in brief, as part of other research articles, and who might want to provide more detailed versions to facilitate use by others.

A survey carried out in 2018 showed that, of more than 2300 users who had followed a protocol published in *Bio-protocol*, 91% (2166 users) were able to successfully reproduce the method they tried.

In this reprint collection, we have selected 26 of the most viewed, downloaded, and cited research protocols related to Microbiology that were published in *Bio-protocol* in 2021.

Hopefully, you will find this collection intriguing and visit <http://www.bio-protocol.org> to check out the entire archive of protocols. Please feel free to email us (eb@bio-protocol.org) with feedback, and please consider contributing a protocol to Bio-protocol in the future.

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On the Cover:

Image from protocol "Histological Methods to Detect Early-stage Plant Defense Responses during Artificial Inoculation of *Lolium perenne* with *Epichloë festucae*."

Evaluation of Toxicity with Brine Shrimp Assay

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Abstract

The *in vivo* toxicity of new metallodrugs either as Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs) or their hydrogels such as with hydroxyethyl-methacrylate (HEMA) (pHEMA@SBAMs or pHEMA@CoMeDs) are evaluated by the brine shrimp assay. Thus individuals of *Artemia salina* larvae are incubated in saline solutions with SBAMs, CoMeDs, pHEMA@SBAMs or pHEMA@CoMeDs or without for 24 h. The toxicity is then determined in terms of the mortality rate of brine shrimp larvae. Brine shrimp assay is a low cost, safe, no required feeding during the assay, while it requiring only a small amount of the tested agent.

Keywords: Bioinorganic Chemistry, Toxicity, *Artemia salina*, Larvae mortality of brine shrimp

This protocol was validated in: Antibiotics (Basel) (2020), DOI: 10.3390/antibiotics9010025

Background

The approval of cisplatin in the clinical treatment of cancer boosted the development of the field of bioinorganic chemistry or medicinal inorganic chemistry. The discovery of new active metallodrugs requires the elucidation of their mode of action. Thus, for example, the targeted delivery of antiproliferative metallodrugs to malignant cells, the activation of inorganic prodrugs and the advent of nanoscience have prompted the scientists to spotlight the toxicity of them (Metzler-Nolte and Guo, 2016). Especially, the research on the design and development of new metallodrugs (either as Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs)), includes their *in vitro* testing against numerous cancerous cell types and their *in vivo* toxicity evaluation towards model organisms (Sainis *et al.*, 2016; Stathopoulou *et al.*, 2018; Banti *et al.*, 2016, 2018, 2019 and 2020; Chrysouli *et al.*, 2018a, 2018b and 2020; Latsis *et al.*, 2018; Milionis *et al.*, 2018; Karetzi *et al.*, 2019; Polychronis *et al.*, 2019; Ketikidis *et al.*, 2020; Rossos *et al.*, 2020). For example: When *Artemia salina* larvae were incubated with the copper(II) complex of amantadine (AdNH_2), with formula $\{\text{[AdNH}_3^+\bullet\text{CuCl}_3]\}$ (CA) (Banti *et al.*, 2020) or the corresponding one of the silver(I) with penicillin G (PenH) $[\text{Ag}(\text{pen})(\text{CH}_3\text{OH})_2]$ (PenAg) (Ketikidis *et al.*, 2020) for 24 h, the percentages of survival of brine shrimp larvae at 30, 60, 90, 120 and 150 μM of CA were (78.3 ± 10.2) , (85.4 ± 6.5) , (87.9 ± 9.6) , (82.6 ± 10.8) and $(76.9\pm11.9)\%$, respectively. The survival rate of brine shrimp larvae at the concentrations of 150 μM , is similar with the corresponding one of the non-treated larvae, suggesting its non toxic behavior. In case of PenAg, the percentage of survival of brine shrimp larvae at 37, 74.5, 150, 220 and 1050 μM are (94.7 ± 2.5) , (87.3 ± 5.0) , (82.6 ± 4.7) , (63.4 ± 6.1) and $(11.0\pm5.0)\%$, respectively, indicating toxicity at the concentration of 1050 μM . Moreover, in the case of the hydrogel which derives by the dispersion of the cluster $\{\text{[Ag}_6(\mu_3\text{-HMNA})_4(\mu_3\text{-MNA})_2]^2\bullet[(\text{Et}_3\text{NH})^+_2\bullet(\text{DMSO})_2\bullet(\text{H}_2\text{O})]\}$ (AGMNA), (H_2MNA = 2-thio-nicotinic acid), in polyhydroxyethyl-methacrylate (pHEMA) (pHEMA@AGMNA-1) (Rossos *et al.*, 2020), no mortality rate of brine shrimp larvae was found, upon their incubation with pHEMA@AGMNA-1 for 2, 4, 6, 8 and 24 h, suggesting the non-toxic behavior of the material.

Artemia salina is a zooplanktonic crustacean found in a variety of seawater systems (lakes, oceans) and it is one of the most popular live foods for many fishes and aquatic invertebrates (Zhu *et al.*, 2018). *A. salina* interacts with the aquatic environment and faces high risk exposure to contaminants (Zhu *et al.*, 2018). The nauplii of the brine shrimp are considered as a simple and suitable model system for acute toxicity tests (Trompeta *et al.*, 2019). The nauplii feature a higher sensitivity to toxic agents compared to the adult Artemia (Trompeta *et al.*, 2019).

A. salina is a popular model organism for toxicological tests, due to its short life-cycle, ease of culture, high offspring production, the commercial availability of its cysts, year-round availability, low cost, safety, no required feeding during the assay, requiring only a small amount of the tested agent (Ates *et al.*, 2013; da Silveira Carvalho *et al.*, 2017; Zhu *et al.*, 2018). Moreover, many endpoints can be selected for toxicological evaluation, including hatching, mortality, swimming, morphology and biomarkers (Živković *et al.*, 2016; Zhu *et al.*, 2018).

The assay correlated with the toxicity data of rodents and humans and shows a good correlation with cytotoxicity tests making these measurements suitable as preliminary results (Živković *et al.*, 2016; da Silveira Carvalho *et al.*, 2017). Artemia species have been used in testing acute toxicity of toxic materials, such as heavy metals and pesticides (Ates *et al.*, 2013), nanoparticles (Zhu *et al.*, 2018), bioactive molecules, natural extracts and metal complexes (da Silveira Carvalho *et al.*, 2017).

Materials and Reagents

Brine shrimp assay

1. Latex gloves (KCWW, Kimberly-Clark, catalog number: 57330)
2. 24-well plate, sterile and tissue-treated (Corning, catalog number: 3526)
3. Pipette tips
4. Brine shrimp eggs (*Artemia salina*) (were purchase from Ocean Nutrition) and the Pure Artemia Cysts are coming from the Great Salt Lake and therefore geographical variations are not affecting the assay
5. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)

6. Sea salt (Tropic Marin)

Equipment

1. 2 L separating funnel with 24/29 joint
2. Stereo microscope Stemi 2000 (ZEISS)
3. Air Pump (Mouse M-106, two outputs 4w)
4. Soft light source such as fluorescent lamps 7w
5. Incubator

Note: Any incubator is fine if it maintains 25°C to 37°C, 50-60% humidity and 12 h day lighting/12 h dark for 24 h.

Software

1. Microsoft Office Excel

Procedure

1. 1 g cysts of *Artemia salina* are soaked in 500 ml natural fresh water for one hour in a 2 L separating funnel. The funnel should not be covered on top.
2. 17 g of sea salt are dissolved in the 500 ml natural freshwater water above.
3. Facilitate the funnel with good aeration using an air pump at room temperature and under continuous illumination for 48 h (Figure 1).



Figure 1. The nauplii incubation apparatus

4. After 48 h hatching, the nauplii released from the egg shells.
5. Collect nauplii at the bright side of the funnel (near the light source) by using a micropipette.
6. Transfer the separated larvae above in a small beaker containing NaCl 0.9%.
7. Introduce an aliquot (0.5 ml) from a small beaker about 10 to 20 nauplii to each well of 24-well plate. The total volume of the well of 24-well plate will be 1ml NaCl 0.9%.
8. Test the metallodrug at three concentrations, with a range of, for example 0.05, 0.5 and 5 μ M with three replicates per concentration. The IC₅₀ value of the metallodrug towards the cancerous cell lines should be in

this range of the tested concentrations. When an antibacterial or a fungicidal compound is tested, then the tested concentration should be the MIC and $2\times$ MIC, $4\times$ MIC values. In the case of hydrogel materials, discs with a diameter of 9 mm were added to each well.

9. The final volume of each well is 1 ml with NaCl 0.9% and the presence of the metallodrug.
10. Maintain the plates at 25°C in an incubator.
11. Examine, after 24 h the brine shrimps, using a stereoscope.
12. Larvae were considered alive if they exhibit internal or external movement during 10 s of observation.
13. Repeat each experiment three times.

Note: During the decapsulation of Artemia cyst, continuous aeration, using an air pump, should be done for proper hatching of the embryo, at 25 °C with simultaneously continuous illumination. For the acceptability of the test, up to 10% of mortality was admitted in the control.

Data analysis

Representative data

Selected icons of hatching eggs, live and dead nauplii brine shrimps which were treated with an agent are shown in Figure 2.

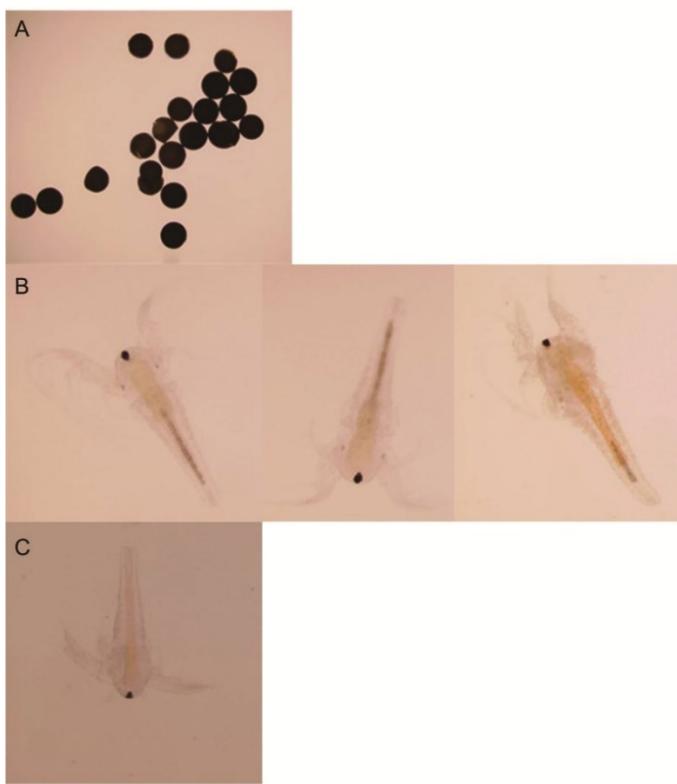


Figure 2. Selected icons of hatching eggs (A), alive (B) and dead (C) nauplii brine shrimps which were treated with a metallodrug

1. Observed larvae, and the dead larvae are considered those that did not exhibit any internal or external movement in 10 s of observation.

2. Count numbers of dead larvae.
3. The (%) mortality of *Artemia salina* larvae was calculated according to Abbott 1987:

$$M(\% \text{ vs. control}) = [(L_C - L_T)/L_C] \times 100$$

where M is mortality; L_C , living nauplii in the control after 24 h; L_T , living nauplii with the tested agent after 24 h.

Acknowledgments

The protocol for *Artemia salina* assay was adapted and modified from a previous study (Syahmiet *et al.*, 2010; da Silveira Carvalho *et al.*, 2017; Rahmanet *et al.*, 2018; Trompeta *et al.*, 2019). It was described in Banti *et al.* (2020), Ketikidis *et al.* (2020) and Rossos *et al.* (2020). This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH-CREATE-INNOVATE (project code: T1EDK-02990) and it has been financially supported by the State Scholarships Foundation (IKY) (Project No. 2019-050-0503-17816), through the Operational Programme "Human Resources Development, Education and Lifelong Learning" in the context of the project "Reinforcement of Postdoctoral Researchers - 2nd Cycle" (MIS-5033021), which is co-financed by Greece and the European Union (European Social Fund- ESF). C.N.B. and S.K.H. are thankful to the COST Action CA17104 "New diagnostic and therapeutic tools against multidrug resistant tumors" members for the stimulating discussions. S.K.H. acknowledges the Oncology Department of Novartis Hellas S.A.C.I. for the financial support (Project No. 82819).

Competing interests

The authors declare no conflicts of interest or competing interests.

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Plant ARGONAUTE Protein Immunopurification for Pathogen Cross Kingdom Small RNA Analysis

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Abstract

Over the last decade, it has been noticed that microbial pathogens and pests deliver small RNA (sRNA) effectors into their host plants to manipulate plant physiology and immunity for infection, known as cross kingdom RNA interference. In this process, fungal and oomycete parasite sRNAs hijack the plant ARGONAUTE (AGO)/RNA-induced silencing complex to post-transcriptionally silence host target genes. We hereby describe the methodological details of how we recovered cross kingdom sRNA effectors of the oomycete pathogen *Hyaloperonospora arabidopsis* during infection of its host plant *Arabidopsis thaliana*. This Bio-protocol contains two parts: first, a detailed description on the procedure of plant AGO/sRNA co-immunopurification and sRNA recovery for Illumina high throughput sequencing analysis. Second, we explain how to perform bioinformatics analysis of sRNA sequence reads using a Galaxy server. In principle, this protocol is suitable to investigate AGO-bound sRNAs from diverse host plants and plant-interacting (micro)organisms.

Keywords: Cross kingdom RNA interference, ARGONAUTE co-immunopurification, Small RNA, Plant-microbe interactions, *Arabidopsis thaliana*, *Hyaloperonospora arabidopsis*, Downy mildew disease

This protocol was validated in: *Elife* (2020), DOI: 10.7554/eLife.56096

Background

Small RNAs (sRNAs) can serve as pathogen effectors that hijack the plant ARGONAUTE (AGO)/RNA-induced silencing complex (RISC) and silence host mRNAs for infection, a virulence mechanism termed cross kingdom RNA interference (Weiberg *et al.*, 2015; Zeng *et al.*, 2019). Profiling the repertoire of sRNAs bound to the plant AGO during infection is the method of choice, to gain a global overview on plant-invasive pathogen sRNAs that might function through the host AGO/RISC. Antibody-based, co-immunopurification (co-IP) of plant AGO/sRNAs, the functional components of a RISC, coupled to sRNA high throughput sequencing is the gold standard to quantify silencing sRNAs in plants (Mi *et al.*, 2008; Montgomery *et al.*, 2008; Carbonell *et al.*, 2012). Such approaches have led to the discovery of specifications for the binding of sRNAs to distinct members of the plant AGO protein family (Mi *et al.*, 2008; Montgomery *et al.*, 2008) and revealed characteristic changes of AGO-bound sRNA profiles according to plant environmental and stress responses (Zhang *et al.*, 2011). In this context, protocols have been published describing how to co-immunopurify plant AGO/sRNAs in order to study AGO-bound, endogenous plant sRNAs under various conditions (Qi and Mi, 2009; Zhao *et al.*, 2012; Carbonell, 2017).

In this bio-protocol, we provide a detailed description of *A. thaliana* AGO1/sRNAs co-IP isolated from *H. arabidopsis*-infected seedlings and the recovery of both plant and pathogen AGO1-bound sRNAs for high throughput sequencing analysis. By this method, we discovered several novel pathogen sRNA effectors as well as plant silencing sRNAs that were responsive to *H. arabidopsis* infection (Dunker *et al.*, 2020). Applying this protocol allowed us to investigate sRNAs bound to other members of the plant AGO family, as well. For instance, we successfully co-immunopurified *A. thaliana* AGO2/sRNAs using a *proAGO2:HA-AGO2* transgenic *A. thaliana* line (Montgomery *et al.*, 2008) in combination with commercial anti-Human influenza hemagglutinin (HA) antibody, and could identify several AGO2-bound *H. arabidopsis* sRNAs (Dunker *et al.*, 2020). Although experimentally validated in the *A. thaliana* system, we propose this protocol being suitable for AGO/sRNAs co-IP and analysis of silencing sRNAs in various plant species and plant-interacting (micro)organisms, given a suitable antibody for AGO co-IP is available and host and microbe genome sequences are known.

Materials and Reagents

Materials

1. Blotting paper (Ahlstrom Munksjö, catalog number: BF4)
2. DNA LoBind® 1.5 mL reaction tubes (Eppendorf, catalog number: 0030108051)
3. Falcon tubes 50 mL and 15 mL (Greiner Bio-One, catalog numbers: 227261 and 188271)
4. Glass pipettes (10 mL)
5. Miracloth (Merck Millipore, catalog number: 475855)
6. Propagation soil substrate (Stender, catalog number: A210)
7. Reaction tubes 1.5 and 2 mL (Sarstedt, catalog numbers: 72.690 and 72.691)
8. 14-day-old *Arabidopsis thaliana* seedlings (ecotype Col-0)
9. *Hyaloperonospora arabidopsis* spores (strain Noco2)

Reagents

Note: If this protocol refers to water, it always implies de-ionized, ultrapure water.

1. Liquid nitrogen
2. Acrylamide/bis-acrylamide solution (Rotiphorese Gel A, Carl Roth, catalog number: 3037)
3. Anti-AGO1 polyclonal antibody (Agrisera, catalog number: AS09 527)
4. Ammonium persulfate (APS) p.a. (Carl Roth, catalog number: 9592)
5. Bromophenol blue Dye (Carl Roth, catalog number: A512)

6. cComplete® protease inhibitor cocktail (Roche, ordered via Sigma-Aldrich, catalog number: 04693116001)
7. Diethyl pyrocarbonate (DEPC, Carl Roth, catalog number: K028)
8. 1,4-Dithiothreitol p.a. (DTT, Carl Roth, catalog number: 6908)
9. Desoxyribonucleotide mix (dNTP, 10 mM each nucleotide type) for molecular biology (New England Biolabs, catalog number: N0447)
10. 96% Ethanol Ph. Eur. (VWR chemicals, catalog number: 20905.296)
11. Ethylenediamine tetraacetic acid (EDTA) disodium salt dehydrate (Gerbu Biotechnik GmbH, catalog number: 1034)
12. Glacial acetic acid (Carl Roth, catalog number: 3738)
13. Glycerol Ph. Eur. (Carl Roth, catalog number: 6967)
14. Glycine p.a. (PanReac/AppliChem, catalog number: 131340)
15. Glycogen RNA grade (Thermo Fisher Scientific, catalog number: R0551)
16. GoTaq® G2 DNA Polymerase (Promega, catalog number: M7841)
17. IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (LI-COR, catalog number: 926-32211)
18. Magnesium chloride (25 mM; for molecular biology) (New England Biolabs, catalog number: B9021)
19. NEBNext® Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, catalog number: E7300)
20. Nonidet P-40 (NP-40, no longer available, the replacement product is IGEPAL CA-630 Sigma-Aldrich, catalog number: I8896)
21. 10 bp O'RangeRuler DNA ladder (Thermo Fisher Scientific, catalog number: SM1313)
22. Potassium chloride (KCl) molecular biology grade (Merck Millipore, catalog number: 529552)
23. Potassium dihydrogen phosphate (KH₂PO₄) p.a. (Carl Roth, catalog number 3904)
24. Protein A agarose (Roche, ordered via Sigma-Aldrich, catalog number: PROTAA-RO)
25. Proteinase K (Thermo Fisher Scientific, catalog number: EO0491)
26. RiboLock® RNase inhibitor (Thermo Fisher Scientific, catalog number: EO0381)
27. ROTI C/I (Chloroform/Isoamyl alcohol mixture, Carl Roth, catalog number: X984)
28. ROTI-Phenol (Carl Roth, catalog number: 0038)
29. ROTI-Phenol/Chloroform/Isoamyl alcohol (Carl Roth, catalog number: A156)
30. Sodium chloride (NaCl) p.a. (Carl Roth, catalog number: 3957)
31. Sodium dodecyl sulfate (SDS) ultrapure (Carl Roth, catalog number: 2326)
32. SuperScript® III (Thermo Fisher Scientific, catalog number: 18080093)
33. Disodium hydrogen phosphate (Na₂HPO₄) p.a. (Carl Roth, T876)
34. Tetramethylethylenediamine (TEMED) for electrophoresis (Carl Roth, catalog number: 2367)
35. 1 M Tris-HCl pH 6.8 stock solution (made from Tris ultrapure, PanReac/AppliChem, catalog number: A1086, pH adjusted with HCl, Carl Roth, catalog number: X896)
36. 1 M Tris-HCl pH 7.5 stock solution (made from Tris ultrapure, PanReac/AppliChem, catalog number: A1086, pH adjusted with HCl, Carl Roth, catalog number: X896)
37. 1 M Tris-HCl pH 8.0 stock solution (made from Tris ultrapure, PanReac/AppliChem, catalog number: A1086, pH adjusted with HCl, Carl Roth, catalog number: X896)
38. 1.5 M Tris-HCl pH 8.8 stock solution (made from Tris ultrapure, PanReac/AppliChem, catalog number: A1086, pH adjusted with HCl, Carl Roth, catalog number: X896)
39. Tris ultrapure (PanReac/AppliChem, catalog number: A1086)
40. Triton X-100 (Carl Roth, catalog number: 6683)
41. Tween 20 (Sigma-Aldrich, catalog number: P9416)
42. 5× Protein SDS loading buffer (see Recipes)
43. 10× Protein SDS running buffer (see Recipes)
44. 10× Protein transfer buffer (see Recipes)
45. 10× PBS pH 7.4 (see Recipes)
46. 50× TAE buffer (see Recipes)
47. DEPC-treated water (see Recipes)
48. IP extraction buffer (see Recipes)
49. IP washing buffer (see Recipes)
50. RNA release buffer (see Recipes)

51. 6% 0.5× TAE gel (see Recipes)
52. 10% 0.5× TAE gel (see Recipes)
53. 8% SDS resolution gel (see Recipes)
54. SDS stacking gel (see Recipes)

Equipment

1. Falcon cooling centrifuge with 15 mL and 50 mL adapters (Eppendorf, model: 5810R, catalog number: 5811000325)
2. Funnel (e.g., Plastic funnel, Carl Roth, catalog number: 2041)
3. Growth chamber
4. Hemocytometer (Neubauer counting chamber, Carl Roth, catalog number: PC73.1)
5. Mortar and pestle
6. PAGE electrophoresis system (Mighty small II system, Hoefer Inc., catalog number: SE250)
7. PCR cycler (FlexCycler, Analytik Jena, succession product is Biometra TOne, Analytik Jena)
8. Standard pipettes of 100-1000 µL, 20-200 µL, 2-20 µL and 1-10 µL (Gilson, catalog numbers: F123602, F123601, F123600, F144802)
9. Pipette controller (Integra Biosciences, ordered via VWR, catalog number: 612-0927)
10. Rolling shaker (TRM 50, IDL GmbH, catalog number: 5200330100)
11. Rotator (AG, FINEPCR, no order number found)
12. Small scissors
13. Spatula
14. Spray unit (Carl Roth, catalog number: YC44.1)
15. Tabletop centrifuge for micro tubes (Eppendorf, model: 5424R, catalog number: 5404000410)
16. Table top mixer (Scientific Industries, model: Vortex Genie 2, catalog number: SI-0236)
17. Thermo shaker with 1.5 mL reaction tube adapter (Eppendorf, model: Thermomixer C, catalog number: 5382000015, can be also used as heat block)
18. Wet blot tank system (Mighty small transfer tank, Hoefer Inc., catalog number: TE22)

Software

Software, bioinformatics tools, and databases:

1. Galaxy server (release 19.01)
2. Illumina Demultiplex (Galaxy Version 1.0.0) (alternative tool for demultiplexing: bcl2fastqc
https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)
3. Clip adaptor sequence (Galaxy Version 1.0.0) (alternative tool for adapter trimming: Trimmomatic [Bolger *et al.*, 2014])
4. Filter FASTQ reads by quality score and length (Galaxy Version 1.0.0)
5. FastQC Read Quality reports (Galaxy Version 0.72)
6. Map with Bowtie for Illumina (Galaxy Version 1.1.0)
7. SAM to FASTQ creates a FASTQ file (Galaxy Version 1.56.1)
8. Filter with SortMeRNA of ribosomal RNAs in metatranscriptomic data (Galaxy Version 2.1b.6)
9. Collapse FASTA sequences (Galaxy Version 1.0.0)
10. TAPIR: target prediction for plant microRNAs (Bonnet *et al.*, 2010)
11. *A. thaliana* TAIR10.0 genome sequence, cDNA sequences
12. *H. arabidopsis* Noks1 (PRJNA298674), Noks1 is a single spore isolate from a Noco2 sample (Bailey *et al.*, 2011)

Procedure

Note: Figure 1 provides an overview scheme of the protocol wet-lab part for your consideration. Plant AGO/sRNA co-immunopurification does not require *in vivo* cross-linking. Before starting, we suggest carefully read the entire protocol. During the procedure, work as quickly as possible, on ice and in a 4°C cold room, when possible, in order to prevent RNA or protein degradation.

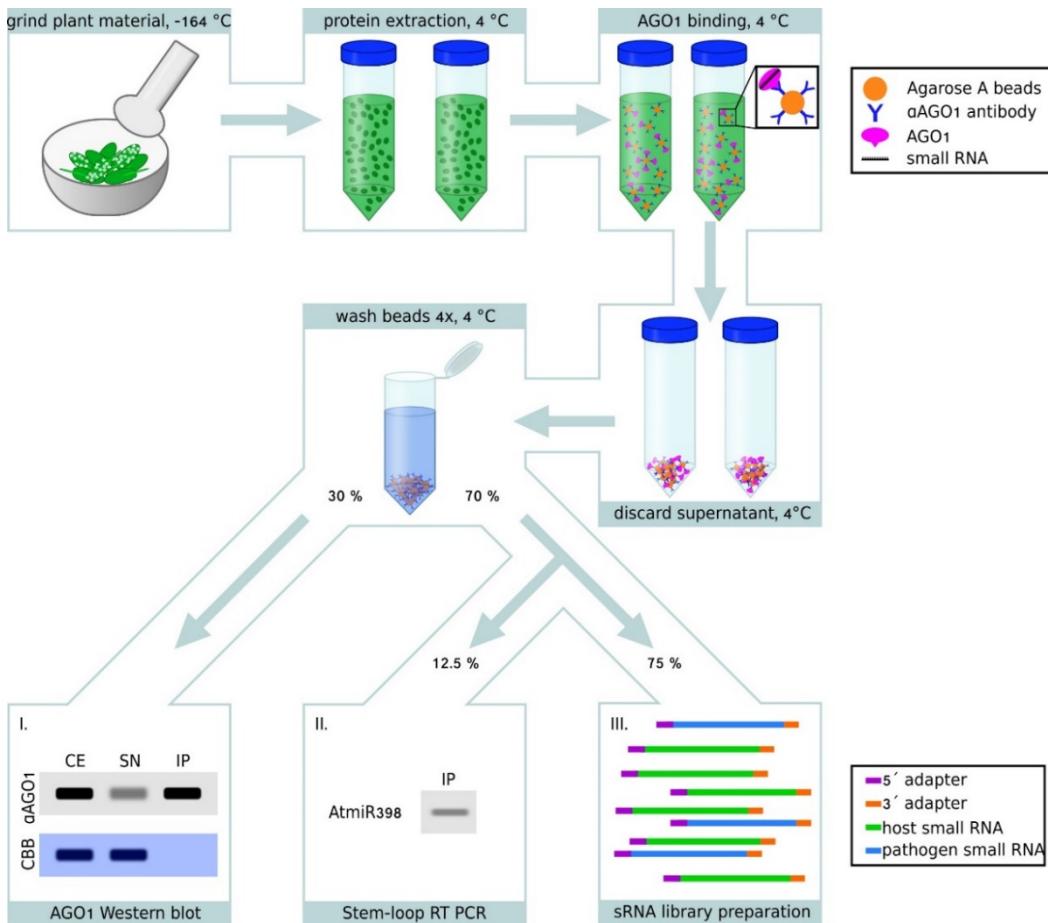


Figure 1. Schematic workflow of plant AGO co-IP for sRNA analysis.

A. thaliana seedling leaves infected with *H. arabidopsis* were ground under liquid nitrogen with pestle and mortar. After adding the IP extraction buffer to the ground leaf material, *A. thaliana* AGO1 proteins were immunopurified (IP) using an AGO1-specific antibody and Protein A agarose beads. After washing the beads, IP samples were used for Western blot analysis and small RNA extraction in a sample volume ratio of 30%/70%, respectively. As a quality control for successful IP, AGO1 was tested in the crude extract (CE), in the supernatant (SN), and in the IP fractions by the Western blot analysis, using total protein reference stained by Coomassie Brilliant Blue (CBB) as a loading control (I.), refer to Figure 2 for a Western blot example. As a quality control for successful co-IP and sRNA extraction, an aliquot (12.5%) of the extracted RNA was used for stem-loop RT PCR amplifying the *A. thaliana* miR398 (AtmiR398) known to bind AGO1 (II.), refer to Figure 3 for a stem-loop RT PCR example. Upon positive results of protein and RNA quality control experiments, the sRNA library was prepared for Illumina-based sequencing (III.).

A. *A. thaliana* seedling inoculation with *H. arabidopsis*

Note: We normally perform dual co-IP experiment of a mock-treated and a pathogen-inoculated sample. However, if more samples are to be prepared, we suggest to process them sequentially in order to prevent protein or RNA degradation.

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degradation. The following inoculation procedure was adapted from Asai et al. (2015).

1. Use 14-day-old *A. thaliana* Col-0 seedlings grown under long-day condition (16 h light/8 h dark) at 22°C and 60% relative humidity in propagation soil substrate. Prepare 10 squared pots (7 × 8 cm) of seedlings per AGO co-IP experiment to obtain enough leaf material.
2. Collect conidiospores of *H. arabidopsis* strain Noco2 from *A. thaliana* Col-0 infected seedlings at 7 days post inoculation using small scissors. Pool collected leaves in a 50 mL Falcon tube avoiding any soil particles.
3. Harvested approximately 2 g of infected fresh leaf material and add 10 mL water to wash off the conidiospores from infected leaves by vigorously shaking the Falcon tube. Filter the conidiospore suspension through a Miracloth and estimate conidiospore concentration by counting conidiospores with a hemocytometer using a light microscope. Adjust the conidiospore suspension to a concentration of 2.5×10^4 spores/ml.
4. Evenly spray 10 mL of 2.5×10^4 spores/ml suspension (or water as mock treatment) on top of *A. thaliana* seedlings using a spray unit.
5. Cover the inoculated or mock-treated seedlings with a transparent lid and seal it, for instance with adhesive tape, to keep high humidity for infection. Incubate the seedlings under long-day conditions (16 h light/8 h dark) at 18°C for 4-7 days.

B. Plant AGO/sRNA co-immunopurification

1. Harvest 5 grams of fresh leaf material at a given time point (in this protocol, we refer to 4 and 7 days post inoculation) with a small pair of scissors and directly transfer leaf material into a pre-cooled mortar containing liquid nitrogen. Grind the leaves into a fine powder with a pre-cooled pestle. Avoid carrying over any soil particles.
2. Once the entire leaf material is powdered, pre-cool a 50 mL Falcon tube and a spatula tip using liquid nitrogen. Transfer the powdered leaf material from the mortar into the Falcon tube using the pre-cooled spatula. Optionally, use a pre-cooled funnel for the transfer. Double-check that all liquid nitrogen has been evaporated before closing the Falcon tube with the plastic lid to avoid explosion of the tube. Place the closed Falcon tube back into liquid nitrogen.
3. Place the Falcon tube from the liquid nitrogen into an ice box and add immediately 20 mL of immunopurification (IP) extraction buffer per 5 grams leaf fresh weight. Due to low temperature of the sample, the IP extraction buffer might freeze at this step. Close the Falcon tube again and thoroughly mix the leaf material in the buffer by placing it on a rolling shaker in a 4°C cold room to let it thaw. Thawing might take up to 50 min.
4. Double-check that thawing is complete, before proceeding.
5. From this step onwards, always keep the sample placed on ice. Spin-down the leaf debris at $3,200 \times g$ for 15 min using a pre-cooled (4°C) centrifuge.
6. In a 4°C cold room, filter the supernatant through a two-layered Miracloth into a new Falcon tube with the help of a glass pipette to remove the cell debris. At this point, split a sample into two aliquots of 10 mL each. Set aside a 200 µL aliquot from this filtered crude extract (CE = input sample) for Western blot analysis. Mix the Western blot sample with 50 µL 5× protein SDS loading buffer and boil it for 5 min at 95°C in a thermo shaker. Store the Western blot sample at -20°C until further use.
7. To continue with AGO co-immunopurification, add 5 µg anti-AGO1 antibody per 5 g original leaf tissue weight as well as 200 µL of protein A agarose beads to the crude extract. Incubate the sample on a rotation wheel for 2 h in a 4°C cold room.

Note: The Protein A agarose beads in the Materials list are provided being pre-equilibrated and ready to use by the manufacturers. Read carefully the manufacturers' manual on how to use this product.

8. Spin-down the sample in a pre-cooled centrifuge at $200 \times g$ and 4°C for 30 s. Take a 200 µL aliquot from the supernatant (SN = unbound fraction) for Western blot analysis. Mix the Western blot sample with 50 µL 5× protein SDS loading buffer and boil it for 5 min at 95°C. Store the Western blot sample at -20°C until

further use.

9. To continue with AGO co-IP, discard the rest of the supernatant. Add 1 mL of ice-cold IP wash buffer to the pelleted beads of the sample, carefully resuspend the beads by pipetting up and down and unify the two sample aliquots into a single 2 mL micro tube.
10. Spin down the sample in a pre-cooled centrifuge at $200 \times g$ and 4°C for 30 s. Remove the supernatant and wash the pelleted beads with 1 mL freshly prepared, ice-cold IP wash buffer by pipetting up and down.
11. Repeat the washing step of the pelleted beads 3 more times.
12. Resuspend the pelleted beads in 1 mL wash buffer and transfer 300 μ L (30% of the sample volume) into a new micro tube for Western blot analysis (IP = immunopurified fraction), keeping the remaining 700 μ L (70% of the sample volume) for RNA extraction. Spin-down both sample aliquots and discard the supernatant.
13. Add 50 μ L of 1× protein SDS loading buffer (prepared from 5× protein SDS loading buffer by diluting with IP wash buffer) to the pelleted beads of the Western blot sample. Boil the beads with the loading buffer for 5 min at 95°C. Store the Western blot sample at -20°C until further use.

C. sRNA recovery from co-immunopurified AGO1 bound to Protein-A agarose beads

Note: Adequate personal protection is mandatory during this part of the protocol since toxic chemicals such as Proteinase K, SDS, phenol, and chloroform are used. Dispose all toxic chemicals according to local legislation.

1. Add 300 μ L of IP wash buffer and resuspend the pelleted beads by pipetting up and down. Add 150 μ L of RNA release buffer. Incubate the sample in a thermo shaker at 300 rpm and 65°C for 15 min.
2. Add 450 μ L water-saturated phenol and mix the samples using a vortexer for 2 min.
3. Separate the phenol-water phases by centrifugation at $10,000 \times g$ at room temperature for 8 min, and transfer the upper aqueous phase including the RNA into a new micro tube.
4. Add 450 μ L of Phenol/Chloroform/Isoamylalcohol (PCI) mixture (25:24:1) to the RNA sample, invert the sample 10 times and separate the PCI-water phases by centrifugation at $10,000 \times g$ and room temperature for 8 min, and transfer the upper aqueous phase containing the RNA into a new micro tube.
5. Repeat the Step C4 two additional times, using Chloroform/Isoamylalcohol mixture (24:1) instead of the PCI. Take great care to avoid carry-over of any traces of the organic phase before starting RNA precipitation. Optionally from this step on, DNA/RNA LoBind® plastic ware can be used to reduce RNA loss.
6. Precipitate the RNA of the sample by adding in the given order 0.1× volume 3 M sodium acetate, 2.5× volume 96% ethanol, and 20 μ g Glycogen (RNA grade). Upon mixing, place the sample at -20°C for a minimum of 1 h for RNA precipitation.

Note: This is a safe stopping point. RNA samples can be stored in 80% ethanol at -20°C.

7. Pellet the RNA by centrifugation at $20,000 \times g$ and 4°C for 30 min, and wash the RNA pellet with 500 μ L 80% ethanol diluted in DEPC-treated water.
8. Pellet the RNA by centrifugation at $20,000 \times g$ and 4°C for 20 min, remove all liquids and air-dry the RNA pellet until ethanol is completely evaporated.

Note: Optionally, the RNA pellet can be dried faster in an open-cap micro tube at 37°C. However, avoid “over-drying” the RNA pellet.

9. Resuspend the RNA pellet in 8 μ L DEPC-treated water. Completely resolve the RNA pellet by incubating the sample for 5 min at 65°C.
10. Store the remaining RNA for the library preparation at -80°C for up to 3 months.

*Note: Optionally, we recommend to perform stem-loop reverse transcription PCR to detect *A. thaliana* microRNA(s) of choice in the AGO co-IP sample as a quality control on the successful recovery of sRNAs before starting the sRNA library cloning (see Figure 3).*

D. Western blot analysis for AGO1 co-immunopurification quality control

Note: The following steps guide through standard Western blot to analyze the three sample types collected throughout the AGO co-immunopurification procedure (crude extract, supernatant, immunopurification). This analysis is essential to confirm efficient AGO purification. In this protocol, Western blots results were visualized on an LI-COR Odyssey detection system. However, any standard protocol for protein identification by Western blot can be used as well.

1. Assemble a protein gel electrophoresis chamber and fill it with protein running buffer. Load 20 µL per sample of the crude extract from Step B6 (CE), the supernatant (SN) after agarose bead collection from Step B8, and of the AGO co-IP (IP) from Step B13 on an 8% polyacrylamide gel. Load 3 µL of a pre-stained protein size marker on the same gel.

Note: For standard protein SDS polyacrylamide gel electrophoresis (PAGE), we used a Rotiphorese acrylamide/bis-acrylamide solution, APS, and TEMED to prepare a discontinuous gel consisting of a 2 mL collection gel at pH 6.8 and a 7 mL separation gel at pH 8.8.

2. Initiate the SDS-PAGE run for 30 min at ~10 V/cm, then increase the voltage to ~17.5 V/cm and let the PAGE run until the 50 kilo-Dalton (kDa) band of the protein size marker reaches the edge of the gel (electrophoresis run takes approximately 90 min).
3. Disassemble the PAGE gel from the electrophoresis chamber and measure the gel size dimensions. Prepare two blotting papers and a PVDF blotting membrane of the measured gel size. Equilibrate the blotting papers and two sponges in the protein transfer buffer for 5 min. Activate the blotting membrane by submerging in 96% ethanol for 1 min and quickly wash-off the ethanol with water. Equilibrate the blotting membrane in the protein transfer buffer until use.

Note: Use a PVDF membrane that is compatible with the Odyssey detection method.

4. Assemble the blotting sandwich in the following order: i) cathode, ii) sponge, iii) blotting paper, iv) polyacrylamide gel, v) PVDF membrane, vi) blotting paper, vii) sponge, viii) anode. Fill the complete blotting tank with protein transfer buffer. Set the amperage to 1 mA cm⁻² of blotting membrane surface and perform blotting of the proteins overnight in a 4°C cold room.
5. On the following day, increase the amperage to 2 mA cm⁻² for 30 min. This step might increase the focus of protein bands.
6. Disassemble the blotting sandwich and roll the membrane with the blotted site to the inside to fit in a 50 mL Falcon tube. Add 10 mL of 5% (v/v) skim fat milk in 1× PBS. Block the membrane for 1 h in a 4°C cold room on a rolling shaker.
7. Discard the blocking solution and quickly wash the blotting membrane with 1× PBS. Add 4 mL of primary antibody solution (anti AtAGO1 1 µg/µL diluted 1:4,000 in 1% milk in a 0.1% PBST buffer) and incubate on a rolling shaker overnight in a 4°C cold room.
8. Wash the blotting membrane 4 times each for 5 min on a rolling shaker in a 4°C cold room with 10 mL of 0.2% PBST buffer.
9. Remove the PBST washing buffer and add 5 mL of secondary antibody solution (anti-rabbit IRdye800 (1 µg/µL) diluted 1:3,000 in 1% milk in a 0.1% PBST with 0.02% SDS). Incubate on a rolling shaker for 1-2 h at room temperature.
10. Wash the blot 4 times each for 10 min on a rolling shaker in a 4°C cold room with 10 mL of 0.2% PBST.
11. Take the membrane out of the Falcon tube and rinse the blotting membrane for 1 min with water and let it dry between two blotting papers. Scan the membrane using an LI-COR Odyssey Scanning device. The band of AGO1 appears at ~130 kDa.

Note: A representative example of a Western blot analysis is displayed in Figure 2. Samples with low AGO signal intensity might still be valid for sRNA library cloning and high throughput sequencing.

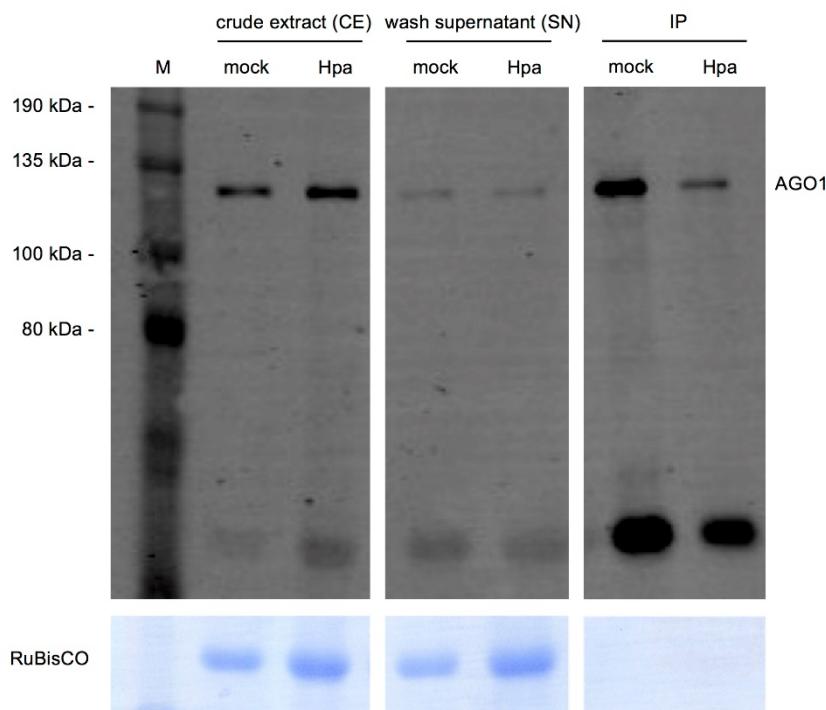


Figure 2. Quality control of AGO1 immunopurification by Western blotting.

Three sample fractions of the AGO1 co-IP experiment were analyzed: crude extract (CE), supernatant (SN), and the IP fraction. These three fractions were analyzed in an *A. thaliana* mock-treated and in an *H. arabidopsis*-infected (Hpa) sample. The top figure shows the detection of *A. thaliana* AGO1 using an AGO1-specific antibody at the expected size of ~130 kDa. Note that AGO1 signals were stronger in IP fractions than in SN. The bottom figure displays the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) signal from a Coomassie Brilliant Blue total protein staining of the Western blot membrane. Note that RuBisCO signals disappeared in the IP fraction. The broad range pre-stained protein marker was used as a protein size marker (M).

E. *A. thaliana* miRNA stem-loop reverse transcription (RT) PCR for AGO/sRNA co-immunopurification quality control

*Note: Before proceeding with library preparation, it is recommended to validate successful RNA co-immunopurification by stem-loop RT PCR. We use the *Arabidopsis* miR398 known to bind AGO1. The protocol directly follows the stem-loop RT PCR protocol as previously described (Varkonyi-Gasic et al., 2007).*

1. Use 1 µL of your eluted RNA (12.5% sample volume) for this quality control assay and pipet it into a PCR micro tube. Add the following components to your RNA sample:

- 1 µL of AtmiR398-specific stem-loop RT primer (1 µM), for the sequence design of stem-loop RT primers, refer to Varkonyi-Gasic et al. (2007)
- 0.5 µL of dNTP mix (10 mM each)
- 8 µL DEPC-treated water

Heat the sample to 65°C for 5 min in a PCR cycler. Cool it down on ice for 1 min.

Add the following components per sample:

- 4 µL 5× first strand SuperScript III reaction buffer
- 2 µL DTT (0.1 M)
- 4 µL MgCl₂ (25 mM)

- 0.25 µL SuperScript III® reverse transcriptase
 - 0.25 µL RiboLock
2. Run the following thermo cycler protocol:
- | | | |
|--------|------|---------------------------|
| step 1 | 16°C | 30 min |
| step 2 | 30°C | 30 s |
| step 3 | 42°C | 20 s |
| step 4 | 50°C | 1 s; back to step 2 (60x) |
| step 5 | 85°C | 10 min |
| step 6 | End | |

Note: The final end-point PCR can be performed with any standard Taq-Polymerase. Below, a protocol using the GoTaq® Polymerase is described.

3. Amplify the reverse transcribed miRNA by end-point PCR. Dilute your stem-loop RT product 1:10 with water as a PCR template.
4. Pipet the following components into a PCR micro tube:
3 µL 5× Green GoTaq® reaction buffer
0.3 µL dNTP mix (10 mM each)
0.5 µL miR398 forward primer (10 µM), for the sequence design of sRNA forward primer, see Varkonyi-Gasic *et al.* (2007).
0.5 µL universal stem-loop reverse primer (10 µM), primer sequence according to Varkonyi-Gasic *et al.* (2007).
9.5 µL water
0.2 µL GoTaq® G2 Polymerase
1 µL stem-loop RT product (1:10 diluted in water)
5. Perform the following thermo cycler protocol:

step 1	94°C	2 min
step 2	94°C	30 s
step 3	60°C	30 s
step 4	72°C	20 s; back to step 2 (36×)
step 5	72°C	2 min
step 6	End	
6. Prepare a 10% 0.5× TAE polyacrylamide gel. Assemble a gel electrophoresis running system. Fill it with 0.5× TAE running buffer.
7. Load 7 µL of each sample in a pocket of the polyacrylamide gel. Pipet 2 µL of 10 bp O'RangeRuler as size marker one the same gel.
8. Run the gel at ~18 V/cm for 90 min to separate PCR products.
9. Disassemble the running chamber and stain the gel with ethidium bromide or any other substitutive DNA dye (e.g., SYBR Gold) for 3 min. If the AGO/sRNA co-immunopurification was successful, you should obtain a PCR band at the size of 69 bp. As an example, a stem-loop RT PCR result of AtmiR398 from an AGO1 co-IP sample is shown in Figure 3.

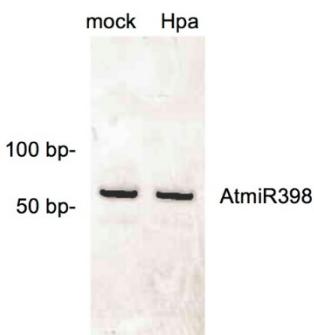


Figure 3. Quality control of sRNAs extracted from AGO1 co-IP samples.

For both mock-treated and *H. arabidopsis*-infected (Hpa) AGO1-co-IP RNA samples, stem-loop RT PCR was performed to detect the AGO1-bound *A. thaliana* AtmiR398. A PCR band of the expected size of 69 base pairs (bp) was visible for both samples. A 10 bp O'RangeRuler DNA ruler was used as a size marker. Depicted samples represent *A. thaliana* leaf materials harvested at 4 days post treatment.

F. sRNA library cloning for Illumina next generation sequencing

Note: We used the NEBNext® Multiplex Small RNA Library Prep Set for Illumina to perform library preparation that is suitable for non-modified as well as modified sRNAs, such as 3' terminal 2'-O-methylation. The protocol is a reprint of the original manufacturer's protocol with slight modifications we applied for AGO co-IP sRNA library cloning. In case library kit protocols are updated by the manufacturer, we recommend to consider the updated instructions.

1. Dilute the required amount of 3' SR adaptor (supplied by NEB kit) 1:2 in nuclease-free water. Mix the following per sample in a 1.5 mL LoBind® reaction tube:
6 µL RNA from the AGO co-IP (75% of the eluted RNA sample volume)
1 µL diluted 3' SR adaptor
2. Incubate the tube for 2 min at 70 °C in a thermo block.
3. Add the following:
10 µL 3' Ligation reaction buffer (2×) (supplied by NEB kit)
3 µL 3' Ligation enzyme mix (supplied by NEB kit)
4. Incubate the sample reaction for 18 h at 16°C.

Note: This prolonged reaction time is recommended to increase the ligation efficiency of methylated sRNAs.

5. Dilute the SR RT primer (supplied by NEB kit) 1:2 in nuclease-free water.
6. Add the following components to the 3'-adaptor/RNA ligation mix (Step F4):
4.5 µL nuclease-free water
1 µL diluted SR RT primer
7. Incubate the mixture for 5 min at 75°C. Transfer the tube to 37°C for 15 min, then to 25°C for 15 min.
8. In the meantime, resuspend the lyophilized 5' SR adaptor (supplied by NEB kit) in 120 µL of nuclease-free water. Store 20 µL aliquots of the adaptor solution at -80°C.
9. Mix the required amount of 5' SR adaptor (supplied by NEB kit) in a 1:1:1 ratio with nuclease-free water and 10 mM ATP in a 200 µL PCR tube. Store remaining adaptor solution at -80°C.
10. Incubate the 5' SR adaptor mix (Step F9) in a thermo cycler for 2 min at 70°C, and place the mix on ice immediately. Use the denatured adaptor mix for the following ligation reaction within 30 min.
11. Add the following components to the reaction tube from Step F7:
1 µL 5' SR adaptor mix (Step F10)
1 µL 10× 5' ligation reaction buffer (supplied by NEB kit)
2.5 µL 5' ligation enzyme mix (supplied by NEB kit)

12. Incubate reaction mix for 1 h at 25°C in a heat block.
13. After this incubation step, add the following components to the reaction and mix well:
8 µL first strand synthesis reaction buffer (supplied by NEB kit)
1 µL murine RNase inhibitor (supplied by NEB kit)
1 µL ProtoScript II reverse transcriptase (supplied by NEB kit)
14. Incubate for 60 min at 50°C. Stop RT reaction at 70°C for 15 min.
15. Add the following components to the cDNA and mix well:
50 µL LongAmp Taq 2× master mix
2.5 µL SR primer for Illumina
2.5 µL Index primer (use a distinct Index primer for each treatment)

Note: As PCR performs better in small reaction volumes, we run 20 µL reaction volumes. After the PCR run was completed, samples were pooled before further procedure.

16. Amplify the library using the following PCR protocol:

step 1	94°C	30 s
step 2	94°C	15 s
step 3	62°C	30 s
step 4	70°C	15 s; back to step 2 (18-22×)
step 5	70°C	5 min
step 6	End	

Note: The expected size of a PCR product representing cloned 21 nucleotides sRNAs is ~140 base pairs. Nevertheless, the PCR product sometimes appears smearable at this point.

G. Purification of the sRNA library for Illumina sequencing

1. Add 0.1× volume 3 M sodium acetate in DEPC-treated water to the PCR reaction in a 1.5 mL LoBind® reaction tube followed by 2.5× volume of ethanol (96%) and 20 µg glycogen. After mixing the solution, incubate the sample for a minimum of 1 hour at -20°C.
2. Prepare a 6% 0.5× TAE polyacrylamide gel.
3. Pellet the sRNA library DNA by centrifugation at 20,000 × g and 4°C for 30 min. Remove the supernatant and add 500 µL of 80% ethanol diluted in DEPC-treated water.
4. Repeat pelleting the sRNA library DNA by centrifugation at 20,000 × g and 4°C for 20 min, remove all liquid from the sample and air-dry the pellet until the ethanol completely evaporated.

Note: Optionally, the DNA pellet can be dried faster in an open-cap micro tube at 37°C, however, avoid “over-drying” the DNA pellet.

5. Resuspend the DNA pellet in 25 µL DEPC-treated water. Incubate the sample for 5 min at 65°C, if you encounter problems with bringing DNA pellet in solution.
6. Mix the eluted library DNA sample with 5 µL of 6× gel loading dye (supplied by NEB kit). Mix the 6× gel loading dye well prior usage.
7. Assemble a gel electrophoresis running system. Fill the electrophoresis tank with 0.5× TAE running buffer.
8. Load 5 µL of the Quick-load pBR322 DNA-MspI digest size marker (supplied by NEB kit) on the same polyacrylamide gel.
9. Use two gel slots per sample by loading 15 µL per well to avoid overloading of the gel lane.
10. Perform gel electrophoresis run with 15 ~V/cm for 60 min; after this running time, the bromophenol blue dye of the loading buffer typically reaches the bottom edge of the gel.
11. Disassemble the gel cast and stain the polyacrylamide gel with ethidium bromide or any other substitutive DNA dye (e.g., SYBR Gold) for 3 min. Under a UV documentation station, cut out the DNA band of the gel at the size of 140-150 base pairs. Avoid any DNA band at ~120 base pairs, as this size usually represents

adaptor dimers.

12. Place the gel piece into a new 1.5 mL DNA LoBind® reaction tube. Use a blue pipette tip to crush the gel piece. Add 250 µL of 1× gel elution buffer (supplied by NEB kit).
13. Elute the sRNA library DNA from the crushed gel pieces by rotating the sample for 2 h at room temperature or overnight in a 4°C cold room.
14. Spin the sample at 10,000 × g and room temperature for 10 min, and transfer the supernatant into a new 1.5 mL DNA LoBind® reaction tube. Try to collect all liquids from the reaction tube.
15. Repeat spinning the sample at 10,000 × g at room temperature for 10 min, and transfer the supernatant into a new 1.5 mL DNA LoBind® reaction tube. At this step, avoid transferring any remaining gel pieces.
16. Add 0.1× volume 3 M sodium acetate in DEPC-treated water, 2.5× volume of 96% ethanol, and 20 µg of glycogen. For DNA precipitation, mix the solution and incubate the sample for a minimum of 30 min at -80°C.
17. Prepare a 6% 0.5× TAE polyacrylamide gel.
18. Pellet library DNA by centrifugation at 20,000 × g and 4°C for 30 min. Discard the supernatant and add 500 µL of 80% ethanol diluted in DEPC-treated water.
19. Pellet the DNA by centrifugation at 20,000 × g and 4°C for 20 min, remove all liquids and air-dry the DNA pellet until ethanol is completely evaporated.

Note: Optionally, the DNA pellet can be dried faster in an open-cap micro tube at 37°C, however, avoid “over-drying” the DNA pellet.

20. Resuspend the DNA pellet in 25 µL DEPC-treated water. Incubate the DNA pellet for 5 min at 65°C, if encounter problems with bringing the pellet in solution.
21. Repeat the gel clean-up of library DNA, following the Steps G6-G18.
22. Store purified library DNA in 80% ethanol at -20°C, until sequencing.

Note: Storage of library DNA is valid for up to 12 months.

23. To proceed with sequencing, precipitate the library DNA by centrifugation at 20,000 × g and 4°C for 20 min, remove all liquids and air-dry the pellet until ethanol is completely evaporated.

Note: In this last step, do not use DEPC-treated water for DNA pellet resuspension as it can interfere with Illumina sequencing; instead use nuclease free water (supplied by the NEB kit).

24. Resuspend the sample in nuclease free water for Illumina HiSeq run.

Note: For sRNA library sequencing, we used an Illumina HiSeq1500 platform in single-end mode with 50 base read length. To obtain sufficient read numbers of pathogen sRNAs collected from infected plant tissue, we recommend to sequence minimum at a depth of 50 million reads per library.

H. sRNA Illumina sequencing analysis

*Note: An overview workflow of the bioinformatics part of this Bio-protocol is shown in Figure 4. sRNA sequencing data were analyzed using a Galaxy Server (Giardine et al., 2005). All tools of the analysis pipeline are also freely available as stand-alone versions, except the Illumina demultiplexing package and the Clip adapter script. As an adequate substitution of these steps, the Illumina bcl2fastqc tool and Trimmomatic can be used, respectively. In the following, a step-by-step description of the bioinformatics pipeline is given for the recovery and identification of *H. arabidopsis* sRNA sequences bound to *A. thaliana* AGO1 during infection, including a target prediction of *A. thaliana* mRNAs using identified *H. arabidopsis* AGO1-bound sRNA sequences. As indicated in Figure 4, a similar analysis can be run to predict plant mRNA targets of endogenous *A. thaliana* small RNAs.*

1. If applicable; demultiplex sRNA libraries using the Illumina Demultiplex or the bcl2fastqc tool by giving the

- library sequence indices.
2. Remove 3'-end adapter sequences from sRNA reads using Clip adapter or the ILLUMINACLIP function of Trimmomatic (Bolger *et al.*, 2014).
 3. Remove low quality reads by setting a minimum Phred quality score of 30.0 and a size range from 19-30 nucleotides using the FASTQ Filter tool (Blankenberg *et al.*, 2010). The FASTQ_filter command is also available as part of the USEARCH package (<https://drive5.com/usearch/features.html>).

Note: Optionally, useful information on sRNA sequencing quality can be found by consulting the FastQC reporter tool at any step of the bioinformatics analysis.

4. To remove *A. thaliana* sRNA reads from the dataset, align the raw reads to the *A. thaliana* reference genome (e.g., TAIR10 for ecotype Col-0) using the Bowtie aligner (Langmead *et al.*, 2009). We recommend allowing one mismatch (-v 1) to map reads to the *A. thaliana* reference genome. By this step, a mapping quality score (MQ) is attributed to individual reads with a score of 255 (aligned) or 0 (unaligned) given in a SAM format output file.
5. Collect reads with MQ = 0, as these reads do not align to the *A. thaliana* reference genome using the Filter_tool (Galaxy Version 1.1.0).
6. Convert the SAM file (unaligned to *A. thaliana*) into a FASTQ file format using the SAM_to_FASTQ tool with SAMtools (Li *et al.*, 2009).
7. Use the Bowtie aligner to align reads from Step H6 to an *H. arabidopsis* Noks1 reference genome (PRJNA298674), allowing zero mismatch (-v 0).
8. Collect reads with MQ = 255, as these read aligned to the *H. arabidopsis* reference genome, using the Filter_tool (Galaxy Version 1.1.0).

Note: We do not recommend to use the Bowtie2 short-read aligner, as this version does not allow binary mapping scores (aligned or unaligned), rather attributes low MQ values to reads with multiple alignment events. Many pathogen sRNAs that we found being loaded into plant AGOs are derived from repetitive DNA, thus such sequences could be easily lost through Bowtie2 quality aware mapping.

9. As a quality control for the successful *A. thaliana* AGO1-co-IP sRNA sequencing, display read counts aligning to the *A. thaliana* reference genome (go back to Step H5 and collect reads with MQ = 255), by read length and 5'-prime nucleotide distribution. *A. thaliana* AGO1 preferentially binds 21 nt sRNAs with 5' prime uracil. This analysis can be run by the FastQC tool, as shown in Figure 5.

*Optionally, remove reads from your dataset (Step H8) that align to ribosomal RNA (rRNA), transfer RNA (tRNA), or small nuclear/nucleolar RNA (snRNA, snoRNA) sequences using the SortMeRNA tool (Kopylova *et al.*, 2012). In particular, rRNA-derived sRNA reads often occur in high abundance in sRNA library sequencing data, if no riboRNA-depletion was performed prior to RNA cloning.*

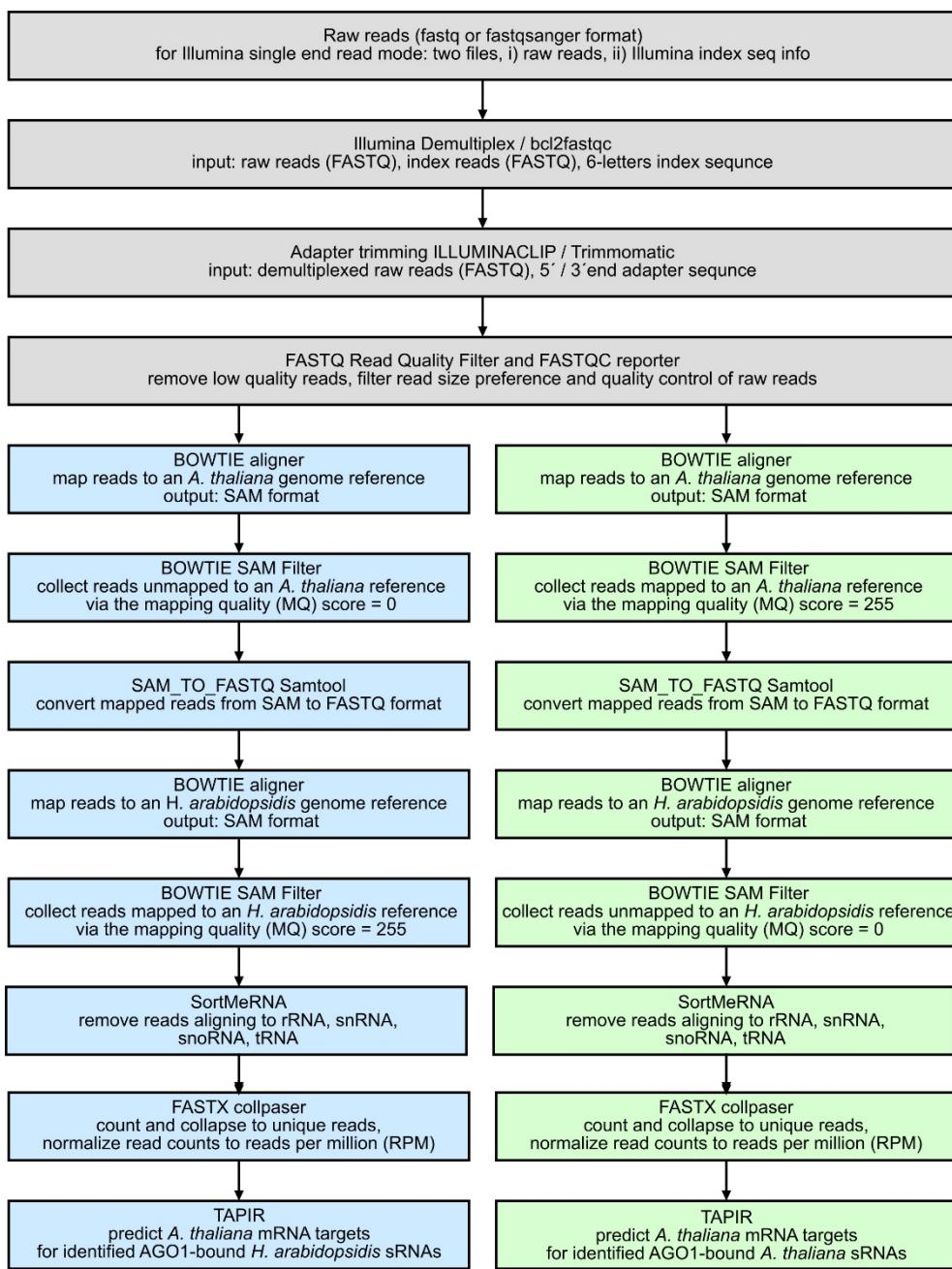


Figure 4. Bioinformatics workflow to analyze AGO1 co-IP sRNA NGS data.

After standard processing of sRNA raw reads, sequences can be grouped into pathogen-derived sRNAs (*H. arabidopsis*) and host plant-derived (*A. thaliana*) sRNAs for further analysis.

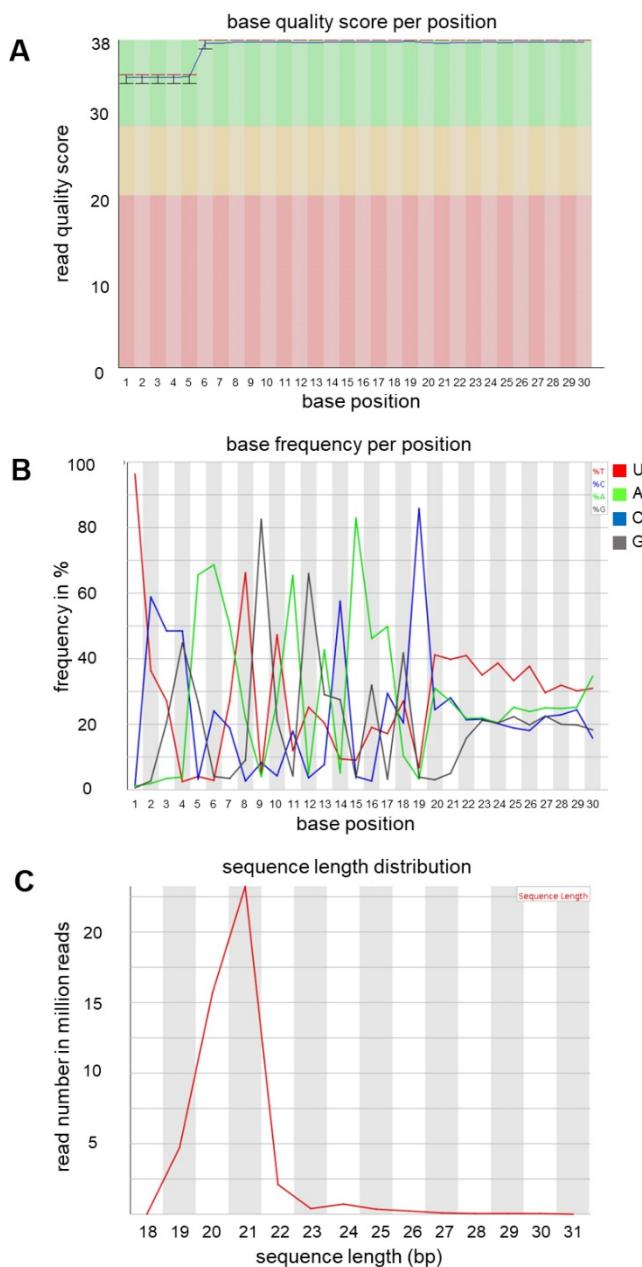


Figure 5. FastQC report of AGO1 co-IP sRNA NGS data.

As plant AGOs are typically associated to distinct sRNA classes, AGO1 co-IP sRNA NGS data reflect some unique features that can be used as a quality control in such experiments. High quality (> 30 Phred score) sRNA reads of the size range of 19-30 bases were collected (A). AGO1-associated sRNAs showed preference to 5' prime Uracil U – in sRNA sequencing data Thymine T (B), and to a size of 21 nucleotides (C). Graphs shown here represent an AGO1 co-IP sample collected from *H. arabidopsis*-infected *A. thaliana* at 4 days post inoculation. The total read number of this sRNA library was 51,089,216.

10. Count and unify sRNA reads to unique sequence tags using a collapse tool, e.g., the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html).
11. Transform read numbers of unique sRNA sequences into reads per million (rpm) by normalizing to the total library read numbers (referring to the organism of interest, here *H. arabidopsis*).

Note: Before starting mRNA target prediction, it can be useful to apply an abundancy filter of small RNA reads.

12. For predicting mRNA targets of candidate sRNAs, several target prediction tools were implemented. We have used the TAPIR tool (Bonnet *et al.*, 2010) to predict *A. thaliana* mRNA targets of *H. arabidopsis* sRNA candidates using an *A. thaliana* cDNA dataset. For target prediction, a maximal score of 4.5 penalizing mismatches in the mRNA/sRNA base pairing, and a maximal free energy ratio of 0.7 were set as thresholds.

Recipes

A. Buffers

1. 5× Protein SDS loading buffer

225 mM Tris-HCl (pH 6.8)
5% (w/v) SDS
50% (v/v) glycerol
0.05% (w/v) bromophenol blue dye
455 mM DTT (added directly before use)

2. 10× Protein SDS running buffer

30 g Tris ultrapure
144 g glycine
10 g SDS
Fill up to 1 L with ultrapure water and stir until salts are dissolved
The pH does not need to be adjusted

3. 10× Protein transfer buffer

30 g Tris ultrapure
144 g glycine
Fill up to 900 mL with ultrapure water and stir until salts are dissolved
Adjust the pH to 8.3 with HCl
Fill up to 1 L with ultrapure water

4. 10× PBS pH 7.4

80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
Fill up to 900 mL with ultrapure water
Adjust the pH to 7.4 with HCl
Fill up to 1 L with ultrapure water
For PBST 0.1% and 0.2% add the respective amount of Tween 20

5. 50× TAE buffer

242 g Tris ultrapure
18.6 g EDTA
a. Dissolve in 950 mL of water
b. Adjust the pH to 8.0 with glacial acetic acid
c. Fill up to 1 L with water

6. DEPC-treated water

Add 1 mL DEPC to 1 L of ultrapure water
Let it stir overnight
Autoclave for 20 min at 121°C

7. IP extraction buffer

20 mM Tris-HCl (pH 7.5)

300 mM NaCl
5 mM MgCl₂
0.5% (v/v) NP-40
5 mM DTT (add directly before use)
1 tablet protease inhibitor/50 mL sample volume (add directly before use)
5 µL RNase inhibitor (40 U)/50 mL sample volume (add directly before use)
Make up to 50 mL with DEPC-treated water

8. IP washing buffer

20 mM Tris-HCl (pH 7.5)
300 mM NaCl
5 mM MgCl₂
0.5% (v/v) Triton X-100
5 mM DTT (add directly before use)
1 tablet protease inhibitor/50 mL sample volume (add directly before use),
Fill up to 50 mL with DEPC-treated water

9. RNA release buffer

100 mM Tris-HCl (prepare from 1 M Tris-HCl pH 7.5)
10 mM EDTA (prepare from 0.5 M EDTA pH 8.0)
300 mM NaCl
2% SDS
1 µg/µL Proteinase K (add directly before use).

B. Polyacrylamide gel recipes (for 10 mL gel volume)

Nucleic acid polyacrylamide gel recipes:

1. 6% 0.5× TAE gel

7.8 mL H₂O
2.0 mL 30% acrylamide/bis-acrylamide solution
0.1 mL 50× TAE buffer
0.1 mL 10% APS
0.008 mL TEMED

2. 10% 0.5× TAE gel

6.5 mL H₂O
3.3 mL 30% acrylamide/bis-acrylamide solution
0.1 mL 10% APS
0.1 mL 50× TAE buffer
0.004 mL TEMED

SDS-protein PAGE gel recipes:

1. 8% SDS resolution gel

4.6 mL H₂O
2.7 mL 30% acrylamide/bis-acrylamide solution
2.5 mL 1.5 M Tris-HCl (pH 8.8)
0.1 mL 10% SDS
0.1 mL 10% APS
0.006 mL TEMED

2. SDS stacking gel

2.1 mL H₂O
0.5 mL 30% acrylamide/bis-acrylamide solution

0.38 mL 1.0 M Tris-HCl (pH 6.8)
0.03 mL 10% SDS
0.03 mL 10% APS
0.003 mL TEMED

Acknowledgments

This protocol has been developed to identify *H. arabidopsis* small RNAs that bind to the *A. thaliana*AGO/RISC during plant infection (Dunker *et al.*, 2020). The authors wish to thank New England Biolabs® for their permission to include the library preparation kit protocol within this protocol. This work was supported by a grant from the Deutsche Forschungsgemeinschaft DFG (WE 5707/1-1) to AW. The funders had no role in the study design, data collection, interpretation, or the decision to publish.

Competing interests

The authors declare no competing interests.

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Rapid Genome Engineering of *Pseudomonas* Assisted by Fluorescent Markers and Tractable Curing of Plasmids

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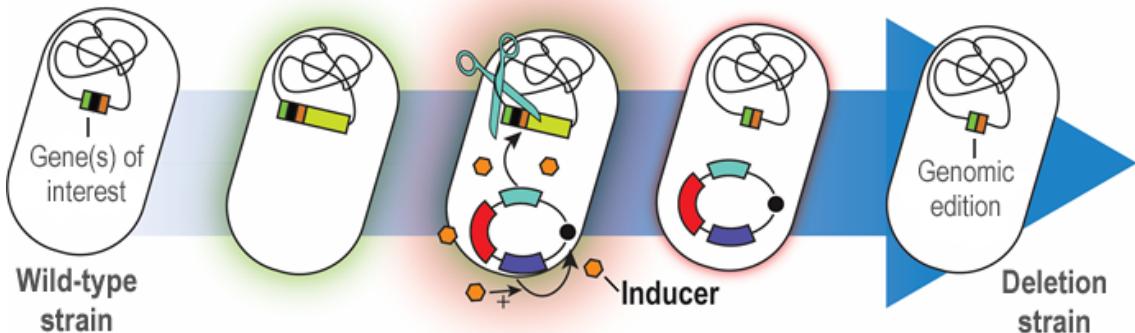
Abstract

Precise genome engineering has become a commonplace technique for metabolic engineering. Also, insertion, deletion and alteration of genes and other functional DNA sequences are essential for understanding and engineering cells. Several techniques have been developed to this end (e.g., CRISPR/Cas-assisted methods, homologous recombination, or λ Red recombineering), yet most of them rely on the use of auxiliary plasmids, which have to be cured after the editing procedure. Temperature-sensitive replicons, counter-selectable markers or repeated passaging of plasmid-bearing cells have been traditionally employed to circumvent this hurdle. While these protocols work reasonably well in some bacteria, they are not applicable for other species or are time consuming and laborious. Here, we present a fast and versatile protocol of fluorescent marker-assisted genome editing in *Pseudomonas putida*, followed by clean curing of auxiliary plasmids through user-controlled plasmid replication. One fluorescent marker facilitates identification of genome-edited colonies, while the second reporter enables detection of plasmid-free bacterial clones. Not only is this protocol the fastest available for *Pseudomonas* species, but it can be easily adapted to any type of genome modifications, including sequence deletions, insertions, and replacements.

Keywords: Genome engineering, Synthetic biology, *Pseudomonas*, Synthetic plasmid replication, Metabolic engineering, Gram-negative bacteria

This protocol was validated in: Metab Eng Commun (2020), DOI: 10.1016/j.mec.2020.e00126

Graphical Abstract:



Rapid genome engineering of *Pseudomonas* with curable plasmids.

Background

Targeted, precise genomic manipulation techniques have considerably advanced the field of microbial engineering. Such methods not only allow for assessing genotype-phenotype relationships, but also enable complex engineering of microbial cell factories. In recent years, CRISPR/Cas9 approaches have paved the way for precise genome engineering in eukaryotes. In bacteria, the use of CRISPR/Cas9 is mainly limited to its value as a counter-selection tool, as bacteria lack non-homologous end-joining to repair the double-strand breaks induced by the Cas9 nuclease. Therefore, engineering efforts in many bacteria rely on homologous recombination (HR) to alter the genome. The advantage of HR is that a broad range of alterations can be introduced in the target genome. Furthermore, it is applicable not only to the so-called model organisms, *e.g.*, *Escherichia coli* and *Saccharomyces cerevisiae*, but also finds wide spread application in non-traditional hosts, *e.g.*, *Pseudomonas* species. In this protocol, we provide a workflow for HR-based genome engineering of *P. putida* – paired with an advanced toolbox that includes several resistance markers – facilitated by the use of fluorescent markers that enable monitoring of every step (Wirth *et al.*, 2020). The presented methodology relies on the co-integration of a suicide plasmid [controlled by the *pir*-dependent origin of replication *ori(R6K)*] at the locus of interest. The co-integration locus is determined by two homologous arms (HAs) on the suicide plasmid, which can be freely chosen by the user to mediate HR. A resolving step forces a second HR event that leads to removal of the plasmid backbone from the genome. This step is triggered by the action of the homing endonuclease I-SceI, acting on two recognition sequences that flank the homologous regions within the backbone of the suicide plasmid. The gene encoding I-SceI is supplied in *trans* from a helper plasmid, introduced into the cells after co-integration of the suicide plasmid. Our recently developed method facilitates rapid curing of this auxiliary plasmid through a synthetic, controllable replication mechanism (Volke *et al.*, 2020) dependent on the presence of 3-methylbenzoic acid (3-mBz). Therefore, plasmid replication can be tightly regulated by the user by merely supplementing or omitting the inducer molecule in the culture medium. Plasmid curing is further aided by the expression of a fluorescent marker from the auxiliary vector, which is compatible with the reporter gene employed in the suicide plasmid. To broaden the use of this method, we developed different versions of the involved plasmids with several antibiotic resistance markers.

Materials and Reagents

Material

1. Pipette tips (1,000 µL, 200 µL, 10 µL) (Sartorius, catalog numbers: 7902020, 790012, 791002)
2. Sterile Petri dishes ($\varnothing = 90$ mm) (HiMedia Laboratories, catalog number: PW001)
3. Eppendorf tubes (Tarsons Products, 1.5 mL, 2.0 mL)
4. 50-mL conical tubes (Sarstedt, catalog number: 62.547.205)
5. Electrocuvettes, 0.1-cm gap for *E. coli* (Bio-Rad, Gene Pulser, catalog number: 165-2089) and 0.2-cm gap for *Pseudomonas* (Bio-Rad, Gene Pulser, catalog number: 165-2086)
6. Sterile 0.2-µm syringe filters (Sigma-Aldrich)

Reagents

1. 3-Methylbenzoic acid (synonym *m*-toluic acid) (3-*m*Bz; Sigma-Aldrich, ReagentPlus, catalog number: T36609)
2. Sucrose (Sigma-Aldrich, Milipore, catalog number: 84100)
3. Lysogeny broth (LB) (Sigma-Aldrich, catalog number: L3522); preparation according to the manufacturer's instructions, storage for up to three weeks at room temperature
4. Agarised LB (Sigma-Aldrich, catalog number: L3147); preparation according to the manufacturer's instruction, storage for up to two months at 4°C
5. SOC medium (Sigma-Aldrich, catalog number: S1797)
6. Kanamycin (TH-Geyer, catalog number: T832.3)
7. Gentamicin (Sigma-Aldrich, catalog number: G1264)
8. Streptomycin (Sigma-Aldrich, catalog number: S6501)
9. Ampicillin (Mitolab, catalog number: K029)
10. Oligonucleotides (Integrated DNA Technologies, Leuven, Belgium)
11. Uracil-specific excision reagent (NEB Biolabs, USER enzyme, catalog number: M5505)
12. DNA polymerase (Thermo Fisher Scientific, *Phusion* U Hot start, catalog number: F555)
13. DNA polymerase reaction mix for colony PCRs including (NEB Biolabs, OneTaq® Hot Start Quick-Load® 2× master mix with standard buffer, catalog number: M0488L)
14. Reagents to prepare chemically competent *E. coli* cells (Zymoresearch, *Mix & Go!*; catalog number: T3001)
15. *Escherichia coli* DH5α λpir [*endA1 hsdR17 glnV44 (supE44) thi-1 recA1 gyrA96 relA1 φ80dlacΔ(lacZ)M15 Δ(lacZYA-argF)U169 zdg-232::Tn10 uidA::pir*+] (Platt et al., 2000)
16. *P. putida* KT2440 (strain ATCC 47054/DSM 6125/NCIMB 11950) (Belda et al., 2016)
17. Sequencing kit (Eurofins, Mix2Seq Kit OVERNIGHT, catalog number: 3094-0ONMSK)
18. Plasmid purification kit (Macherey-Nagel, NucleoSpin Plasmid, catalog number: 740588)
19. Gel and PCR Clean-up Kit (Macherey-Nagel, NucleoSpin™ Gel and PCR Clean-up Kit, catalog number: 740588)
20. Optional: *Dpn*I (Thermo Fisher Scientific, FastDigest *Dpn*I, catalog number: FD1703)
21. Agarose (Bio-Rad, Certified Molecular Biology Agarose, catalog number: 1613102); prepare at 1% (w/v) in 1× TAE buffer for gel electrophoresis (use microwave heating to dissolve). Can be stored at 60 °C to keep molten for immediate use
22. Fluorescent nucleic acid staining solution (Intronbio, Red safe, catalog number: 21141)
23. DNA ladder (Thermo Fisher Scientific, GeneRuler 1 kB, catalog number: SM0314)
24. 3-Methylbenzoic acid (3-*m*Bz) solution (500 mM) (see Recipes)
25. Sucrose solution (300 mM) (see Recipes)
26. Polymerase chain reaction (PCR) reagents (see Recipes)
27. Electro-competent *Pseudomonas* cells (see Recipes)
28. Antibiotic stock solutions (see Recipes)

Equipment

1. Electroporator (Bio-Rad, MicroPulser, catalog number: 1652100)
2. Transilluminator (Thermo Fisher Scientific, Safe Imager 2.0 Blue Light, catalog number: G6600)
3. Table centrifuge, used for 1.5- and 2-mL reaction tubes (VWR, model: Microstar 17R, catalog number: 521-1647)
4. Table centrifuge, used for 50-ml reaction tubes (Thermo Fisher Scientific, model: Heraeus Multifuge X1R, catalog number: 75004250) with rotor (Thermo Fisher Scientific, model: TX-400, catalog number: 75003181) and buckets (Thermo Fisher Scientific, catalog number: 75003655) and adaptors (Thermo Fisher Scientific, catalog number: 75003683)
5. Termoblock (Eppendorf, model: Thermo Mixer C, catalog number: 5382000015)
6. pH-meter (Thermo Fisher Scientific, model: FE150pH, catalog number: S35924)
7. Agarose chamber, gelcaster, combs and power supply (Bio-Rad, Mini-sub Cell GT system & Power pack, catalog number: 1645050)
8. Gel visualization (Bio-Rad, Gel Doc XR+ Gel Documentation System, catalog number: 1708195EDU)
9. PCR thermocycler (Eppendorf, Mastercycler Nexus X2 Thermocycler, catalog number: 6336000015)

Software

1. AMUSER [<http://www.cbs.dtu.dk/services/AMUSER> (Genee *et al.*, 2015)]
2. DNA sequence design tool for example Benchling or Geneious (Biomatter Ltd.)

Procedure

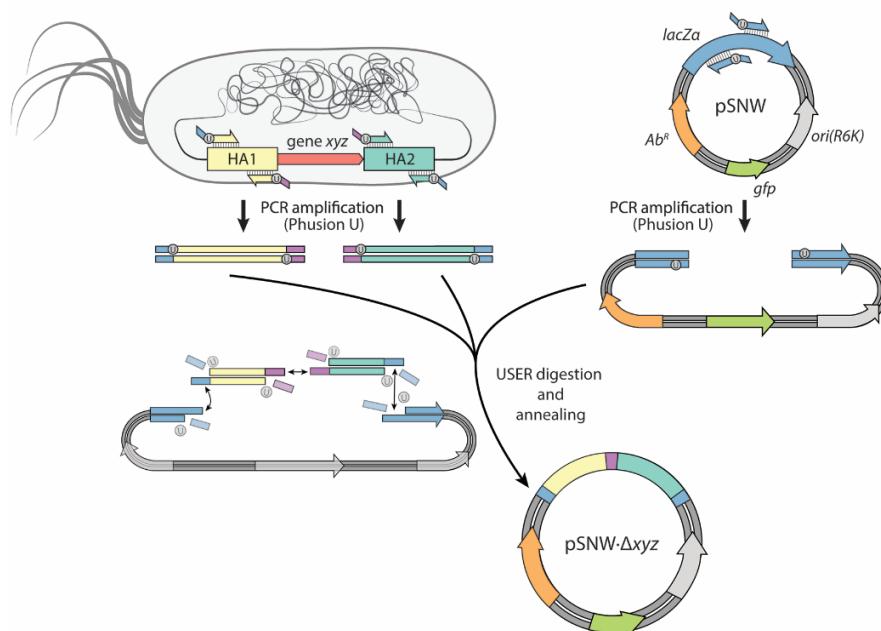


Figure 1. Designing integration vectors for genome manipulations.

In this example, we illustrate the construction of a plasmid for deleting gene(s) *xyz* on the chromosome of *P. putida*. First, the pSNW plasmid backbone as well as the two homology arms flanking *xyz* (HA1 and HA2) are amplified with *Phusion U* DNA polymerase, thereby introducing complementary, homologous overhangs containing a single

deoxyuridine nucleoside (dU). The amplicons are combined and digested with the uracil excision reagent (*USER*), leading to the removal of dU to create single-stranded overhangs. After a subsequent annealing of the fragments, the plasmid is delivered into chemical-competent *E. coli* DH5 α λ pir cells.

A. Cloning of integration vector

A suicide plasmid for specific genomic manipulations is composed of a universal pSNW plasmid backbone (see **Table 1**), an upstream homology arm (HA1), *if applicable*: a DNA sequence to be inserted at the target site, and a downstream homology arm (HA2) (**Figure 1**). Each of these fragments is PCR-amplified. Thus, three to four pairs of primers are required per construct.

Table 1. Suicide plasmids for co-integration

Plasmid	Antibiotic resistance	Fluorescent marker
pSNW2	Kanamycin	GFP
pSNW4	Streptomycin	GFP
pSNW5	Tetracycline	GFP
pSNW6	Gentamicin	GFP

1. Identify the sequence of the two regions flanking the target gene or locus on the chromosome of *P. putida*. The upstream homologous arm (HA1, see **Figure 1**) spans from 500-700 bp upstream of the chromosomal target to the first base of the sequence that is to be deleted or edited, or the integration site. The downstream homologous arm (HA2, see **Figure 1**) conversely starts after the last base of the chromosomal target and ends 500-700 bp further downstream.

Tip: We recommend leaving the STA2RT and STOP codons of the target gene intact and deleting only the interjacent sequence to minimize the chance of polar effects due to gene deletions and to avoid the potential creation of toxic, truncated polypeptides.

2. Design primers for the construction of the application-specific pSNW plasmid
Plasmids from the pSNW series can be linearized for USER cloning using the same pair of primers for every procedure (pSNW-USER_F: 5'-AGT CGA CCU GCA GGC ATG CAA GCT TCT-3', and pSNW-USER_R: 5'-AGG ATC UAG AGG ATC CCC GGG TAC CG-3'; dU residues highlighted in red in the primer sequences), so that only insert-specific primers need to be designed for every genomic manipulation.

Open the AMUSER online software (<http://www.cbs.dtu.dk/services/AMUSER/>) and enter the sequence of each fragment comprising the pSNW plasmid insert identified in *STEP 1* (HA1, HA2, and, *if applicable*: integration fragment) in FASTA format (including a header preceded by “>” and a DNA sequence). At *STEP 2: Output construct*, select *linear* and click on *Submit query*. The primer sequences from the *AMUSER report* can directly be used for the amplification of the fragments. Add the motif **5'-AGA TCC U-3'** as the primer overhang to the forward primer of HA1, and **5'-AGG TCG ACU-3'** as overhang to the reverse primer of HR2. These two overhangs match the ones that have been used to linearize vector pSNW.

Tip: Standard cloning procedures can be also established for Gibson Assembly or Golden Gate cloning to streamline the process.

One standard set of primers can be used to check for the correct insert size after construction of the pSNW derivative in colony PCR, as well as to sequence the insert region. The two primers bind within the pSNW backbone immediately upstream (pSNW_seq_F: 5'-TGT AAA ACG ACG GCC AGT-3') and downstream (pSNW_seq_R: 5'-CTT TAC ACT TTA TGC TTC CGG-3') of the insert region, respectively.

3. Design primers for genotyping after the genome manipulation
Design one pair of primers binding within a range of 50 bp upstream of HA1 and 50 bp downstream of

HA2 in the genome, respectively. These primers are used to test for the cells' genotype after the recombination step via integration of vector pSNW. For small insertions or modifications that do not alter the total length of the sequence comprising both HAs, an additional primer that specifically binds within the insertion/modification is helpful to identify the engineered genotype.

4. Amplification of regions by PCR

We recommend using a ‘touchdown’ temperature protocol (Don *et al.*, 1991) for each PCR amplification (see **Table 2**), as it circumvents the needs for optimizing the annealing temperature and leads to higher yields and specificity (Korbie and Mattick, 2008). Amplify vector pSNW using 5 ng of plasmid, mini-prep isolated to increase template concentration and purity, and primers pSNW-USER_F and pSNW-USER_R using the *Phusion U* Hot Start DNA polymerase (see **Recipe 3**). Use the temperature protocol illustrated in **Table 2**, with an elongation time of 3 min. Adopt the same temperature protocol to amplify each of the fragments constituting the pSNW insert, only adjusting the duration of the extension steps according to the amplicon lengths. Utilise purified genomic *Pseudomonas* DNA with the appropriate primer pairs to generate the HR1 and HR2 fragments. Perform, if needed, additional PCRs to generate the DNA fragments required for insertions.

Tip: We recommend to gel-purify the linearized pSNW fragment using a gel and PCR clean-up kit according to the manufacturer's instructions. By using the purified product as template for future PCRs, digestion with *DpnI* to remove circular plasmids (i.e., template) can be omitted. We further recommend generating a large amount of linearized pSNW vector in several parallel PCRs for repeated usage in USER cloning.

Table 2. Touchdown temperature protocol for Phusion U PCR

Initial denaturation	Touchdown phase			Enrichment phase			Hold
	Denaturation	Annealing	Extension	Denaturation	Annealing	Extension	
98°C	98°C	68°C (-1°C per cycle)	72°C	98°C	58°C	72°C	12°C
30 s	10 s	15 s	20 s/kb	10 s	15 s	20 s/kb	∞
	10 cycles			27 cycles			

5. Check PCRs on agarose gel

Analyse a 3-μL aliquot of each PCR by agarose gel electrophoresis [1% (w/v) agarose and 1× fluorescent nucleic acid gel stain in 1× TAE buffer] to verify the correct amplification of the fragments. The relative amplicon concentrations can be semi-quantitatively estimated from the intensities of their bands. If agarose gel electrophoresis reveals the presence of non-specific by-products, the desired bands have to be excised from the gel and purified prior to cloning using a gel and PCR clean-up kit according to the manufacturer's instructions. If the product appears clean, the PCR reaction can be directly used in the assembly reaction.

6. USER reaction

In a PCR tube, combine equimolar amounts of all fragments (HR regions, insert and backbone) in 10 μL with a total amount of around 50 ng. Add 1 μL of 1 U μL⁻¹ USER enzyme. Set up a thermocycler and run a reaction program as follows: *deoxyuracil excision*: 30 min at 37°C; *annealing 1*: decrease from 28°C to 18°C with -2°C per step of each 3 min; *annealing 2*: 10 min and hold at 10°C.

!!! If a plasmid was used as template for the amplification of one of the fragments that contains the same antibiotic resistance as the employed pSNW vector, add 0.5 μL of FastDigest *DpnI* to the reaction mix prior to incubation at 37°C !!!

7. Transformation of *E. coli* DH5α λpir cells

Transform 50 μL (or 100 μL) aliquots of chemically-competent *E. coli* DH5α λpir cells with 5 μL (or 10 μL) of the assembly reaction from the previous step. To prepare competent cells and transform them with

assembled plasmids, we recommend using the *Mix & Go! E. coli* Transformation Buffer Set (Zymo Research) and the associated protocol. After delivery of the plasmid into the cells (via heat-shock or incubation, see *Mix & Go!* Instruction manual), add 1 ml of SOC medium and incubate for 1 h at 37°C with gentle shaking at 200-300 rpm. Pellet the cells at 10,000 × g for 1 min, resuspend in 50-100 µL of SOC medium and plate the suspension onto LB agar plates supplemented with the respective antibiotic for the pSNW plasmid.

!!! Critical: It is essential to use an *E. coli* strain harboring the λ phage-derived *pir* gene for replication the of *ori(RK6)* !!!

Tip: If a circular plasmid was used as template in the PCR for linearization of vector pGNW and if the reaction was directly employed for the assembly reaction (rather than using a gel-purified plasmid), spread the transformed *E. coli* DH5α λpir cells on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 µg ml⁻¹. The disruption of the pSNW-borne lacZα sequence then allows for the identification of *E. coli* colonies harbouring “empty” template plasmids via their blue colour in contrast to white colonies, carrying a pSNW with insert.

8. Check for correct insert size

Perform a colony PCR (using OneTaq® 2× Master Mix) on eight to ten colonies that show green fluorescence under blue-light exposure (inspect plate with transformed *E. coli* DH5α λpir on a blue-light transilluminator) with primers pSNW_seq_F and pSNW_seq_R. To this end, prepare 50 µL of a 1× master mix by mixing 25 µL of OneTaq® 2× Master Mix with 23 µL DNase-free water and 1 µL of each primer (scale up if necessary). Transfer 6 µL of the 1× master mix into PCR tubes and add a small amount of biomass from the *E. coli* colonies grown on the culture plate. Run a PCR with the following temperature protocol (Table 3, adjust the elongation time according to the expected insert size).

!!! For *E. coli*, *P. putida* and many other bacteria, small amounts of biomass from colonies can be directly transferred with a pipette tip or inoculation loop to the reaction mix (avoid transferring agar from the plate to the reaction mixture, as it will inhibit the amplification). For some bacteria, it might be necessary to boil biomass in water and dilute before colony PCR for good amplification results !!!

Table 3. Touchdown temperature protocol for OneTaq colony PCR

Initial denaturation	Touchdown phase			Enrichment phase			Hold
	Denaturation	Annealing	Extension	Denaturation	Annealing	Extension	
94 °C	94°C	62°C (-1°C per cycle)	68°C	94°C	52°C	68°C	12°C
30 s	20 s	30 s	1 min/kb	20 s	30 s	1 min/kb	∞
	10 cycles			27 cycles			

9. Check colony PCRs on agarose gel

Analyse a 3-µL aliquot of each PCR by agarose gel electrophoresis [1% (w/v) agarose in 1× TAE buffer] to verify the correct insert size.

10. Verify insert sequence integrity by sequencing

If no band other than that of the expected size is visible in the agarose gel, the reaction sample can directly be sent out for sequencing. For this, mix 0.5 µL of the PCR reaction with 14.5 µL of DNase-free water and 2 µL of the primer pSNW_seq_F or pSNW_seq_R (one sequencing sample for each primer and each *E. coli* clone) in a barcoded tube from a Mix2Seq kit and send the tubes for sequencing. If non-specific bands appeared in the agarose gel, inoculate 3-5 mL of LB medium (add the corresponding antibiotic) in 50-mL centrifuge tubes with three individual clones that were tested for a correct insert size. Incubate the cultures at 37°C for 12-18 h in a shaking incubator at 180-250 rpm (depending on the type of incubator). Purify plasmid DNA from the *E. coli* cultures and send the purified plasmid DNA for sequencing. Use a sequence-verified plasmid for the subsequent procedure in *P. putida*.

B. Integration of suicide plasmid into desired genome locus

1. Transformation of *P. putida*

Follow the recipe for preparing electro-competent *Pseudomonas* cells (**Recipe 4**). Electroporate the cells with 300-500 ng of the previously constructed pSNW plasmid (see Procedure A). **Figure 2** presents an overview of the procedure described in this section.

Tip: If the strain has a low competence or the plasmid has a low integration efficiency, triparental conjugation can be used instead of electroporation. See protocols in Martínez-García and de Lorenzo (2011), Sánchez-Pascuala et al. (2017) and Durante-Rodríguez et al. (2018) for further details.

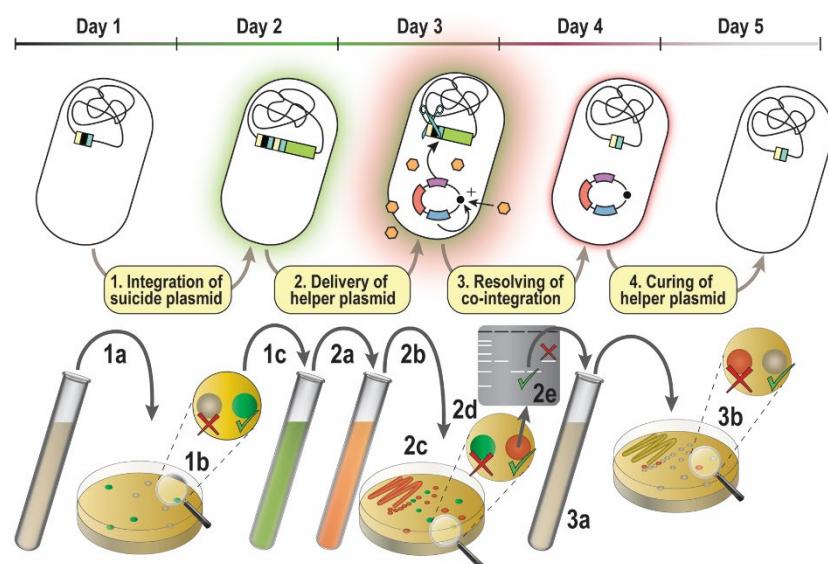


Figure 2. Overview of the genome editing procedure.

1a) *P. putida* is transformed with a suicide plasmid from the pSNW series. 1b) Genomic integration of the vector is enforced by plating on selective medium. 1c) The genome integration is confirmed *via* green fluorescence of antibiotic-resistant colonies. 2a) Confirmed co-integrants are propagated in selective medium and electroporated with an auxiliary plasmid from the pQURE series. 2b) The co-integration is resolved *via* the expression of *I-SceI* from pQURE. 2c) Single colonies from suspension are obtained by dilution-streaking and 2d) the resolution of co-integration is confirmed by the absence of GFP-fluorescence. 2e) The genotype of selected colonies is confirmed by colony PCR; colonies can be either revertants or carry the desired genome alteration. 3a) A modified clone is propagated in non-selective medium. 3b) Single colonies are obtained by dilution-streaking and clones that have lost pQURE are identified by the absence of red fluorescence.

2. Plating

Plate on LB agar with either kanamycin for pSNW2; gentamicin for pSNW6 or streptomycin for pSNW4 and incubate the plates at 30°C for ~16 h. The growth conditions may have to be adjusted depending on the strain to be edited. Grown colonies should be big enough to identify fluorescence on a blue-light transilluminator and to be easily picked with an inoculation loop.

3. Fluorescence check on transilluminator

Colonies with the suicide plasmid integrated should display green fluorescence (**Figure 3A**). Mark several fluorescent colonies for the further steps.

Tip: Fluorescence increases significantly over time after plating due to maturation of the fluorophore. Therefore, plates can be incubated for an extended time at room temperature or at a lower temperature

(4 °C) to increase the signal. Usually, all antibiotic-resistant colonies should display green fluorescence. The (unlikely) absence of fluorescence may indicate the presence of contaminations.

C. Resolving of co-integration

- Propagation of confirmed co-integrand and transformation with pQURE plasmid

Pick up to six co-integrand colonies, verified by fluorescence, and transfer their biomass together into 10 ml of LB medium in a 50-ml centrifuge tube with the appropriate antibiotic. Incubate at 30°C for ~16 h with shaking. Prepare the culture for electroporation, and use ~10 ng of pQURE plasmid (**Table 4**). Add 3-mBz at 2 mM directly to the recovery medium.

!!! Choose a pQURE variant whose antibiotic resistance is compatible with the employed suicide plasmid and organism (i.e., no natural resistance and not the same antibiotic resistance marker as used for pSNW) !!!

!!! Critical: Omit the antibiotic used to select for pSNW co-integration from now on, otherwise mutations within pSNW are selected which rules out its successful resolution !!!

Table 4. Plasmids for resolving of co-integration

Plasmid	Antibiotic resistance	Fluorescent marker
pQURE·1	Ampicillin	RFP
pQURE·2	Kanamycin	RFP
pQURE·6	Gentamicin	RFP

- Induce double strand break and homologous recombination event

Continue culturing the cells for 2 h before adding the appropriate antibiotic to select for pQURE. After 1-3 additional hours, either (i) plate a volume of 70 µL onto LB agar supplemented with 2 mM 3-mBz and the appropriate antibiotic to select for pQURE and incubate the plate overnight at 30°C, and/or (ii) pass the suspension on to 10 ml of selective LB medium with 2 mM 3-mBz and continue incubation at 30°C with shaking for about 16 h.

Tip: The longer the growth in selective liquid medium, the higher the resolving efficiency and the lower the chance to obtain genetically heterogeneous colonies containing revertants and cells with the desired genetic alteration. The resolving efficiency can vary strongly between different loci.

- Isolating single mutants

If (ii) was chosen in the previous step, perform a dilution streak of the culture on selective (for pQURE) solid medium with 2 mM 3-mBz.

Tip: You want dozens of isolated colonies. You can perform multiple dilution streaks from one culture on a single plate to increase the numbers of isolated colonies.

- Check fluorescence on a blue-light transilluminator

Resolved colonies do not display green fluorescence and should appear red under blue-light exposure (from the RFP encoded on pQURE, **Figure 3B** and **3C**). In general, the majority of colonies will be resolved. Mark several colonies without green fluorescence for further analysis.

*!!! Critical: 3-mBz in the plates is slowly degraded by some microbes, e.g., *P. putida*, leading to a brown coloration of the colonies and the surrounding medium. The brown pigment produced conceals the colonies' fluorescence after prolonged storage (several days to weeks) and therefore, the colonies should be marked immediately !!!*

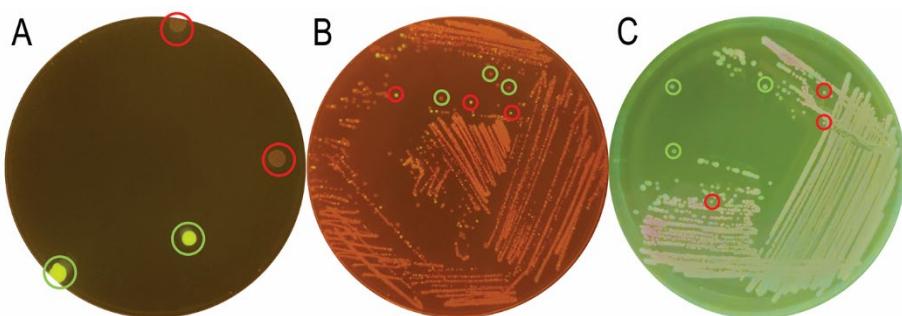


Figure 3. Exemplary plates for different steps of the genome engineering process.

A. Colonies of *P. putida* after co-integration of the suicide plasmid display green fluorescence (marked with green circles) after ~24 h of incubation. B. Resolved culture after ~18 h of incubation. Most colonies display a slightly red colour (marked with green circles). Only a few show green fluorescence (marked with a red circle). The latter still contain the co-integrated plasmid. C. Culture plate with cured colonies after ~24 h of incubation. Several resolved colonies without red fluorescence (highlighted with green circles) are kept for further analysis.

5. Check genome editing in resolved colonies

Check the locus of interest with the genotyping primers designed in A3 via colony PCR (see A8 and Table 3) for at least eight colonies. For genomic deletions or insertions, the amplicon size differs for the two genotypes and thus allows for their identification. For small insertions or sequence replacements, a primer specific for the modified sequence together with the external primer binding on the opposite strand will yield a product only for modified genotypes. The ratio of revertants, *i.e.*, unmodified genotype, and modified genotype can vary greatly, depending on either a fitness advantage for one of the two genotypes or the sequence context of the modified locus.

Tip: An aliquot of the amplified region can be directly used for sequencing. For this, add 0.5 μ l of the PCR product to 14.5 μ l of DNase-free water and 2 μ l of the respective primer used for its amplification. For genomic manipulations that cause a severe detriment to the bacterium's growth, the use of CRISPR/Cas9 counter selection can facilitate in obtaining the desired mutant genotype [please refer to Wirth et al. (2020) and Volke et al. (2020) for details].

D. Curing of auxiliary plasmid

1. Propagate a colony with the desired genotype

Inoculate 10 ml of LB medium **without** any additives (neither antibiotics nor 3-mBz) with biomass from a single colony. Incubate the culture at 30°C until it reaches stationary phase (typically overnight).

Tip: It is advantageous to use as little biomass as possible to allow for a maximum number of replications of the cells in liquid LB medium.

2. Selection of the cured strain

Make a dilution streak of the culture on an LB plate without antibiotic or 3-mBz to obtain isolated colonies. Incubate the plate at 30°C for ~16 h. Plasmid-cured strains can be identified on a blue-light transilluminator by the absence of red fluorescence.

Tips:

- The wavelengths created by the blue-light transilluminator are not optimal for RFP visualization. Thus, high amounts of matured RFP are needed. Since the maturation time of RFP is long, incubation of the plate at 4–8°C for several hours is helpful to increase the fluorescence intensity. The signal

matures during the course of several days and can even be seen with the naked eye after incubating the plate for 2 days at 4°C.

- b. Use of a light source with a wavelength closer to the excitation maximum of RFP (i.e., 558 nm) increases the fluorescence signal significantly, enabling the selection of colonies with relatively low RFP levels.
3. Storage of cured, engineered strain
Inoculate 10 ml of LB medium with the cured colony. After incubation at 30 °C for ~16 h, you can either store the strain or proceed working with it.

Recipes

1. 3-Methylbenzoic acid (3-mBz) solution (500 mM)

Ingredients	Per 250 mL
3-Methylbenzoic acid	17 g
a.	Add 3-mBz into 200 mL of distilled water
b.	Stir vigorously with a magnetic stirrer and insert pH-probe
c.	Add drop-wise 5 M NaOH solution until all 3-mBz is dissolved and the pH reaches 7.0
d.	Fill up to 250 mL with distilled water
e.	Sterile-filter the solution with a 0.2-μm filter

Note: Solution can be stored at room temperature for several months. 3-mBz is added in a 1:500-ratio to culture media immediately before use. Let molten agar cool down to ~50°C before adding 3-mBz and immediately cast plates.

2. Sucrose solution (300 mM)

Ingredients	Per 500 mL
Sucrose	52.3 g
a.	Add sucrose into 400 mL of distilled water
b.	Stir vigorously with magnetic stirrer until dissolved
c.	Fill up with distilled water to 500 mL
d.	Sterile-filter with a 0.2-μm filter

Note: Solution can be stored at room temperature for several months. Aliquoting (50 ml) is recommended to prevent contamination.

3. Polymerase chain reaction (PCR)

Ingredients	Per 50 μL [μL]
Phusion U	0.5
dNTPs	1
Buffer HF	10
DMSO	1.5
Oligonucleotides	2.5 each
Template	10 ng plasmid (or biomass)
H ₂ O	Fill up to 50 μL

4. Electro-competent *Pseudomonas* cells

- a. Inoculate 10 mL of LB medium with a single colony in a 50-mL tube.
- b. Grow the culture for ~16 h at 30°C in a shaking incubator (200-250 rpm).

Note: All of the following steps are performed at room temperature, if not otherwise stated.

- c. Harvest the cells by centrifugation ($4,000 \times g$; 10 min, room temperature), resuspend in 1 mL of 300 mM sucrose and transfer into a microreaction tube (1.5 mL or 2 mL).
- d. Pellet the cells by centrifugation ($10,000 \times g$, 1 min, room temperature) and resuspend in 1 mL of the sucrose solution.
- e. Repeat the washing step (resuspension and centrifugation) two times. Finally, resuspend the pellet in 400 μ L of 300 mM sucrose. The cell suspension can be stored for several hours on ice before use or transformed directly.
- f. Mix 100 μ L of cell suspension with 10 ng or >200 ng DNA for replicated or suicide plasmids, respectively. Electroporate (2.5 kV, 25 μ F capacitance, 200 Ω resistance), immediately add 1 mL of LB medium and transfer the suspension into a test tube (recommended: 14-ml round bottom test tube). Recover the cells for 2 h at 30°C with shaking at 200-250 rpm.

Note: In the following steps, you can either plate the culture to obtain single transformants or transfer the suspension into selective liquid medium, if isolated clones are not needed.

5. Antibiotic stock solutions

Kanamycin: Dissolve 0.5 g of kanamycin in 10 mL of MilliQ water.

Gentamicin: Dissolve 0.1 g of gentamicin in 10 mL of MilliQ water.

Streptomycin: Dissolve 0.5 g of streptomycin in 10 mL of MilliQ water.

Note: Sterile-filter the solution and aliquot at volumes of 1 ml. Solutions can be stored at -20°C for several months. Add antibiotic stock solution to liquid and solid media immediately before use in a 1:1,000 ratio. Let molten agar cool down to ~50°C before adding antibiotics and immediately cast plates.

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Competing interests

The authors declare there are no potential conflicts of interest.

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Phytophthora infestans (Late blight) Infection Assay in a Detached Leaf of Potato

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Abstract

Phytophthora infestans is a hemibiotroph oomycete that primarily infects potato and tomato. It infects stems, leaves, and tubers and fruits of potato and tomato. High throughput and reproducible infection assays are prerequisites to find sources of resistance in any crop. In this protocol, we describe a detached leaf assay (DLA) for conducting the virulence assay of *P. infestans* in potato leaves. A late blight infection assay using a potato detached leaf is a semi-high throughput assay in which hundreds of plants can be screened in a laboratory setting.

Keywords: *Phytophthora infestans*, Potato, Detached leaf assay, Late blight

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Background

Potato (*Solanum tuberosum* L.) is one of the most important non-cereal food crops in terms of food and nutritional value (Zhang *et al.*, 2017). Late blight of potato caused by the oomycete pathogen *P. infestans* is one of the most devastating potato diseases in the world and is the most important yield-limiting factor in potato production (Haverkort *et al.*, 2008 and 2016; Fisher *et al.*, 2012). Breeding for late blight resistance is considered an important factor to fight against this disease. For this purpose, identification of novel sources of resistance in the available germplasm is a crucial step. Several testing methods such as field tests, whole plant assays, and detached leaf assays (DLA) have been developed. DLA provides increased infection and potato leaves showing more susceptibility to *P. infestans* than the field and whole plant assays (Stewart, 1990; Vleeshouwers *et al.*, 1999; Vossen *et al.*, 2016). Since the genotype of the host and pathogen are generally static in infection assays, observed differences in susceptibility among testing methods are likely due to variation in environmental conditions. DLA is suited for identification of qualitative resistance available in the germplasm which is typically a qualitative trait governed by a single or a few disease resistant (R) genes.

Materials and Reagents

1. Conical tubes
2. Combitips advanced 1.0 mL (Eppendorf, catalog number: 0030089430)
3. Cheese cloth
4. Petri Dish (100 mm × 15 mm) (Fisher Scientific, catalog number: FB0875713)
5. Parafilm (PM-996)
6. Nunc square standard height bioassay dishes (Thermo Fisher ScientificTM, catalog number: 240835)
7. Heavy-duty paper towels (Uline, catalog number: S-13631BLU)
8. Spreader (Universal Medical, catalog number: HS86655)
9. *P. infestans* isolate, US-23
10. Sterile water
11. 70% ethanol
12. Potato genotypes, obtained from U.S. Potato GenBank (Sturgeon Bay, WI)
13. Fertilizer (Peters' Professional 20-10-20 Peat Lite special)
14. Growing media
 - a. For *P. infestans* Rye A media (Caten and Jinks, 1968) (60 g of Rye grain, 15 g of agar and 20 g of sucrose for 1 L of distilled water, for detail please see Reference)
 - b. For plants soil mixtures (All-purpose mix BM1, Berger)

Equipment

1. Eppendorf Repeater E3 (Eppendorf, catalog number: 4987000398)
2. Biological safety cabinet
3. Light microscope
4. Scanner (Epson, model: Perfection V700 Photo)
5. Scalpel
6. Hemocytometer
7. Lamp
8. Cold room at 4°C or refrigerator
9. Incubator (Fisher Scientific, catalog number: 97-990E)
10. 10 × 10 cm pots
11. 15 × 15 cm pots

Software

1. ImageJ

Procedure

A. Growing conditions of plants and collection of leaves

1. Germinate potato seeds or plant tubers, cuttings or tissue culture plantlets in a greenhouse in soil-less potting mix (BM1, Berger) in a 10×10 cm pot.
2. Transplant the seedlings after two weeks into the 15×15 cm pots.
3. Maintain a temperature of 22°C during the day (a photoperiod of 17.5 h) and 20°C during the night (dark) in the greenhouse.
4. To maintain the health of plants, irrigate regularly however avoid excessive moisture, fertilize once a week and spray against insects and foliar disease, when necessary. However, avoid spraying with fungicides for one week before inoculations.

B. Growing conditions of *P. infestans*

1. Grow *P. infestans* isolate US-23 on a Rye A media plate (Figure 1) at 18°C . This plate can be used as a master plate for next 3 months for the further sub-culture.



Figure 1. *P. infestans* growth in Rye A plates after 12 days

2. Cut three plugs (5 mm) of *P. infestans* isolate, US-23 from Rye A media.
3. Place these three plugs in a new Rye A media plate in a triangular fashion (Figure 1).
4. Seal the plates properly with parafilm and incubate in the dark at 18°C .
5. Keep the plates facing downward to avoid any moisture development on the plugs.

C. Inoculum preparation

1. Harvest the sporangia from a 10-14-day old Rye A cultured plate (Figure 1) by flooding the plate with 5 mL ice-cold sterilized water (to expedite the release of zoospores) and mix properly with a spreader.
2. Keep the plate at 4°C for 2 to 4 h to release zoospores.
3. Harvest the zoospores by filtering the liquid from each plate through two layers of cheesecloth (to remove mycelia) and dilute in 20 mL of ice-cold sterilized water.

4. Count the motile zoospores using a hemocytometer under a microscope and adjust the concentration to 50,000 zoospores per mL of water. Generally, we make 100 mL final solution (zoospores and water) with 50,000 zoospores from a 10-14-day old Rye cultured plate.

D. Infection assay

1. Collect healthy, full-grown compound leaves having at least three leaflets from 5-8-week-old plants if available. Care should be taken not to collect old (start showing sign of yellowing) or very young leaves (not-fully developed).
2. Set up a bioassay plate lined with wet Uline heavy-duty paper towels (Figure 2).



Figure 2. Late blight infection assay conducted on bioassay plate lined with wet paper towel.
Top cover was removed to take the picture.

3. Drop inoculate the abaxial side of each leaflet with 10 µL droplets (4 to 6 droplets per leaflet) of inoculum (50,000 zoospores per mL) with an Eppendorf repeater.
4. Keep the bioassay plates in a room with natural light at a temperature of 21°C.

E. Symptom monitoring

1. The first symptoms can be observed 3 days after inoculation as black/brown lesions, sporulation, and a water-soaked area at the point of pathogen inoculation.
2. Subsequently, symptoms enlarge and cover the whole leaf in the case of highly susceptible genotypes after 5 days.
3. Assess the leaves visually for the appearance of symptoms using a 1-9 scale (Karki *et al.*, 2020) (Figure 3) or quantified by using ImageJ software (Rueden *et al.*, 2017), 5 days after inoculation.

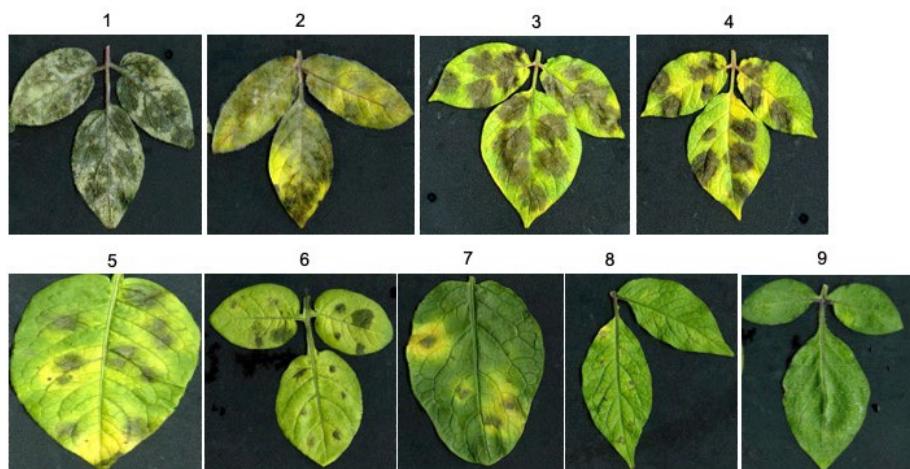


Figure 3. The 1-9 scale used to evaluate the late blight infection.

The mean leaf area covered with blackish/brown lesions, sporulation, and water-soaking was calculated using ImageJ and assigned a value based on this area. 1 = $\geq 90\%$, 2 = 81-90%, 3 = 71-80%, 4 = 61-70%, 5 = 41-60%, 6 = 21-40%, 7 = 10-20% with cell death at the point of inoculation, 8 = $\leq 10\%$ with cell death at the point of inoculation, and 9 = 0% infection, no visible symptoms, clean leaves.

F. Quantification of diseased area

1. Obtain a digital image of the leaf using a flatbed scanner or camera (Image type: 24-bit color, Resolution: 300 dpi).
2. Start ImageJ and open the image (File > Open...).
3. Select the ‘Straight Line’ tool.
4. Draw a line to an object of known size (e.g., coin or ruler).
5. Go to ‘Analyze > Set scale and enter the preferred unit of length (e.g., cm, mm, or inches) and the known distance of the line from Step 4. Click OK.
6. Use the ‘Paintbrush tool’ to highlight the diseased area of the leaf. Double-click the paintbrush icon to set the brush options, including brush width. Care should be taken to select only the diseased area and not other injuries.
7. Go to ‘Image > Type > 8 bit’.
8. Go to ‘Image > Adjust > Threshold (to get red leaf images).
9. Select the dark background checkbox, and slide both bars to the very right (255 value). The selected infected area should be red and the remainder of the leaf photo should be in greyscale.
10. Close the threshold settings window.
11. Use ‘Freehand selection’ tool and trace the outline of the whole leaf area.
12. Go to ‘Analyze > Set Measurement’ and select only the ‘Area’ and ‘Area fraction’ checkboxes, deselect any other checkboxes, and click OK.
13. Go to ‘Analyze > Measure’ or press Ctrl+M to measure the diseased leaf area. A separate ‘Results Window’ will pop up showing the whole area and the % area, which represent the surface area of the outlined leaf in square units defined in step 5 and the percent infected area of the leaf, respectively.
14. When closing the ‘Results Window’, you will have the option to save the results.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

This protocol describes details of the late blight infection assay used in experiments previously published by Karki *et al.* (2020). Work was supported by the USDA NIFA/NSF Plant Biotic Interactions Program award number: 2018-67014-28488.

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An Imaging Flow Cytometry Method to Measure Citrullination of H4 Histone as a Read-out for Neutrophil Extracellular Traps Formation

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Abstract

The formation of neutrophil extracellular traps (NETs) is thought to play a critical role in infections and propagating sterile inflammation. Histone citrullination is an essential and early step in NETs formation, detectable prior to the formation of the hallmark extracellular DNA-scaffolded strands. In addition to the classical microscopy method, new technologies are being developed for studies of NETs and their detection, both for research and clinical purposes. Classical microscopy studies of NETs are subjective, low throughput and semi-quantitative, and limited in their ability to capture the early steps. We have developed this novel Imaging Flow Cytometry (IFC) method that specifically identifies and quantifies citrullination of histone H4 as a NETs marker and its relationship with other alterations at nuclear and cellular level. These include nuclear decondensation and super-condensation, multi-lobulated nuclei versus 1-lobe nuclei and cell membrane damage. NETs markers can be quantified following variable periods of treatment with NETs inducers, prior to the formation of the specific extracellular DNA-scaffolded strands. Because these high throughput image-based cell analysis features can be performed with statistical rigor, this protocol is suited for both experimental and clinical applications as well as clinical evaluations of NETosis as a biomarker.

Keywords: NETs detection, NETs quantification, Imaging flow cytometry, NETosis, Histone H4 citrullination, Nuclear decondensation

This protocol was validated in: Front Immunol (2020), DOI: 10.3389/fimmu.2020.01335

Background

Activated neutrophils are rapidly recruited to sites of infection and injury; they contribute to host defense against pathogens and inflammation. About two decades ago, it was observed for the first time that in response to pathogens, a fraction of the neutrophil population can undergo a distinct type of cellular death, different from apoptosis or necrosis. This involves early chromatin decondensation, co-localization of the nuclear and granular compartments and finally, release of this mix into the environment. The extracellular DNA-scaffolded strands anchoring nuclear components (such as histones) or cellular constituents, some with strong anti-pathogen properties like elastase or myeloperoxidase (MPO), are known as “neutrophil extracellular traps” or NETs and have effective antimicrobial activity (Brinkmann *et al.*, 2004). More recent evidence shows that NETs also associate with non-infectious conditions including cancers, systemic lupus erythematosus, sickle cell disease, atherosclerosis, and thrombosis (Jorch and Kubes, 2017). It is yet not understood whether the NETs, or any of their components (cell-free DNA or histones) contribute to the pathology of these disorders as drivers, or whether they are by-products of an unbalanced immune response. NETs might also function as biomarkers in certain diseases and provide information regarding the efficacy of a treatment regimen (Barnado *et al.*, 2016). NETs-associated components such as elastase or MPO are present in the plasma of individuals with autoimmune diseases and also in infections, which complicates the specificity of these molecules as biomarkers. In this light there is a great need for new methodologies for NETs characterization that offers consistent criteria between studies, and are reliable for both mechanistic and clinical applications.

Other NETs detection and quantification methods are being developed in addition to the classical widely used immunofluorescence microscopy technique. These include use of confocal microscopy that provides information on the structure of the NETs (Santos *et al.*, 2018), ELISA-based assay to quantify the citrullinated histone 3 (H3cit) in human plasma (Thalin *et al.*, 2017), high throughput live detection of NETosis- and apoptosis-related nuclear changes using membrane-permeable and -impermeable nuclear dyes in human neutrophils (Gupta *et al.*, 2018), quantification of free chromatin (predominantly associated with elastase) in human whole blood by using microfluidics (Muldur *et al.*, 2018). Several studies monitored NETs components (H3cit, MPO and extracellular DNA) in human and mice by using conventional flow cytometry (Gavillet *et al.*, 2015; Masuda *et al.*, 2017; Lee *et al.*, 2018; Zharkova *et al.*, 2019). While this technique allows high-throughput analysis it also does have noteworthy limitations, regarding the choice of the probes, the preparation of the samples and the way gating impacts final conclusions (Manda-Handzlik *et al.*, 2016; Masuda *et al.*, 2017). On the other hand, while Imaging Flow Cytometry (IFC) does allow high-throughput analysis, it also combines visualization and analysis features from both conventional flow cytometry and fluorescence microscopy (Basiji, 2016). NETs-associated morphological nuclear changes (*i.e.*, chromatin decondensation and DNA trails) and whether and to what degree MPO co-localized with the nuclear compartment were investigated with IFC (Zhao *et al.*, 2015; Ginley *et al.*, 2017; Pelletier *et al.*, 2017). Thus, different markers and different methods are currently employed to detect and quantify NETs *in vitro*. PAD4-mediated histone citrullination has been long designated as a hallmark of NETs formation, and thus a desirable marker to follow. More recent reports, however, predominantly in the mouse model, suggest that PAD4-mediated citrullination of histone 3 (H3cit) is stimulus-dependent (Neeli and Radic, 2013; Claushuis *et al.*, 2018; Guiducci *et al.*, 2018; Liang *et al.*, 2018) and therefore the utility of this site as a NETs marker might be limited.

Here, we describe in detail a novel IFC protocol that allows specific detection and quantification of citrullination of histone 4 (H4cit3) as a NETs marker in whole neutrophils, prior to the release of the DNA and the cytoplasmic protein strands into the extracellular space. Other analysis parameters that look at nuclear and cellular morphological changes (nuclear decondensation and supercondensation, multi-lobulated nuclei and cell membrane damage) can bring additional information on the behavior of the analyzed neutrophil population. To establish our methodology we determined the responses of healthy human neutrophils treated for 5 different periods of time (between 2 min and 60 min) with NETs agonists: pharmacological inducers, PMA and calcium ionophore (a well-known inducer of histone citrullination, and for our experimental conditions, the positive control), Hemin (an inducer present under hemolytic conditions) and LPS and IL-8 (inducers associated with infectious pathogens). We used *in vitro* fluorescence microscopy to confirm the formation of DNA-elastase-MPO strands in healthy neutrophils treated with the stimuli used for the IFC tests (Barbu *et al.*, 2020). We further validated this technique in untreated and Hemin-treated neutrophils from healthy donors and patients with sickle cell disease at steady state (Barbu *et al.*, 2019).

The increased sensitivity and objective analysis make this methodology highly suitable for NETs detection in
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both research and clinical studies, that explore mechanistic answers or possible therapeutic strategies.

This protocol has 4 major parts: one-step neutrophil purification, induction of NETosis, specific staining, IFC acquisition and analysis (as highlighted in Figures 1A and 1C), with the first 3 steps requiring 3 days and up to one week to complete all steps.

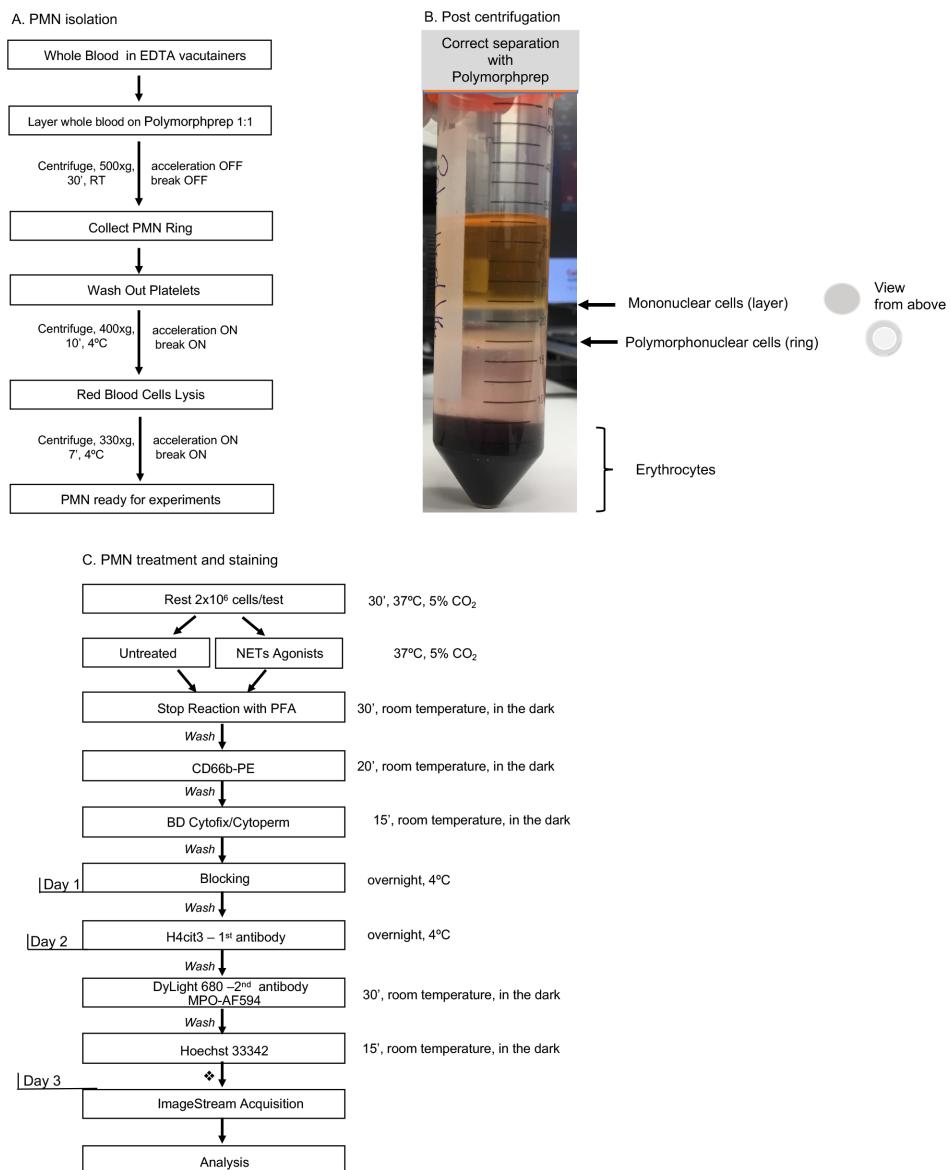


Figure 1. Day-by-day description of the major steps for specific detection of NETosis markers by Imaging Flow Cytometry.

A. One-step polymorphonuclear (PMN) cells isolation from whole blood with Polymorphprep medium; with this method neutrophils purity should be consistently above 80%. Total processing time: 90 min. **B.** An example of correct separation of whole blood by using Polymorphprep gradient medium. **C.** Detailed description of polymorphonuclear cells treatment and staining procedure. A time recommendation is not included for the neutrophil stimulation step as researchers should choose an appropriate treatment time according to their experimental purpose. Each washing step might need up to 20 min to complete, depending on the number of samples processed. This time should be taken into account while calculating total time required to complete all

steps per day. ♦ Short final wash followed by samples storage at 4°C. Total stimulation and staining processing time for Day 1: 4+ h.

Materials and Reagents

1. See Through, Lavender, 10.0 V, 16 × 100 mm, Plastic Tube with BD Hemograd Closure (BD, catalog number: BD366643)
2. Polypropylene Conical Tubes, 50 mL (Fisher Scientific, Corning, catalog number: 07-203-510)
3. Centrifuge tubes, 5 mL, conical bottom, sterile (Benchmark Scientific, catalog number: C1005-T5-ST)
4. Centrifuge tubes 1.5 mL, mixed neon colors (USA Scientific, catalog number: 1415-1448)
5. Rapid flow sterile disposable filter units with PES membrane, capacity 500 mL, pore size 0.45 µm (Thermo Scientific Nalgene, catalog number: 09-740-63E)
6. Aluminum foil
7. Polymorphprep gradient medium (Cosmo Bio USA, catalog number: AXS-1114683)

Note: Polymorphprep should be stored at room temperature, in the dark. Long term exposure to light affects its efficacy.

8. RPMI-1640 without L-glutamine (Lonza, catalog number: 12-167F)
9. L-glutamine 200 mM (Thermo Fisher, catalog number: 25030-081)

Note: Aliquot in one-time use fractions and store at -80°C. Thaw in water bath at 37°C to dissolve the white sediment, then add to the RPMI to make the complete neutrophil medium.

10. Stock 32% Paraformaldehyde (PFA) Aqueous Solution, EM Grade (Science Services, catalog number: E15714). Once open use within days
11. Ultrapure 0.5 M EDTA pH 8.0 (Thermo Fisher, Invitrogen, catalog number: 15575020)
12. DPBS 1×, no calcium chloride, no magnesium chloride (Gibco, catalog number: 141190-136)
13. Cell Culture Grade Water (Corning, catalog number: 25-055-CV)
14. Bovine Serum Albumin, heat shock fraction, protease free, pH 7.0, ≥98% (Millipore Sigma, catalog number A3294)
15. Potassium Chloride Granular (Mallinckrodt, catalog number: 6858)
16. Dimethyl Sulfoxide (DMSO), ≥99.5% (GC), plant cell culture tested (Sigma-Aldrich, catalog number: D4540)
17. Hemin from bovine, ≥90% (Millipore Sigma, catalog number: 9039)

Note: Prepare a 40 mM stock in DMSO, aliquot in single use fractions and store at -20°C. Mix well when preparing the working solutions. Add a DMSO-only control with the tested samples.

18. PMA, for use in molecular biology applications, ≥99%, HPLC (Millipore Sigma, catalog number: P1585)

Note: Prepare a stock solution in DMSO and freeze single use aliquots at -20°C.

19. LPS-EB, Ultrapure, *E. coli* 0111:B4 (InvivoGen, catalog number: tlrl-3pelps)

Note: Prepare a stock solution in ddH₂O, aliquot and freeze at -20°C. Limit the thaw-freeze cycles to three.

20. Recombinant Human IL-8 (CXCL7, 77aa) (Peprotech, catalog number: 200-08)
21. Calcium Ionophore A23187 ≥98% (TLC), powder (Millipore Sigma, catalog number: C7522)
22. Anti-Human CD66b-PE, clone G10F5 (Biolegend, catalog number: 305106)
23. Rabbit polyclonal anti-histone H4, citrulline 3 (H4cit3, Millipore Sigma, catalog number: 07-596) (see Notes)

24. Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight 680 (Thermo Fisher, catalog number: 35568) (see Notes)
25. MPO Polyclonal Antibody, AlexaFluor 594 conjugated (Bioss Antibodies, catalog number: bs-4943R-A594)
26. Hoechst 33342 (BD Pharmingen, catalog number: 561908)
27. Gelatin from Porcine Skin, powder, Type A, suitable for cell culture (Millipore Sigma, catalog number: G1890)

Note: Prepare 2% stock and keep it at 4°C for up to 6 months. Open in sterile hood only. Monitor for signs of bacterial or fungal contamination.

28. BD Cytofix/Cytoperm kit (BD Biosciences, catalog number: 554722). Use only the Cytofix/Cytoperm solution in this protocol
29. Wash Buffer (see Recipes)
30. Blocking buffer (see Recipes)
31. Paraformaldehyde 8% working solution (see Recipes)
32. Porcine skin gelatin stock 2% (see Recipes)
33. 0.6 M KCl (see Recipes)
34. Neutrophils complete medium (see Recipes)

Equipment

1. Water bath at 37°C
2. LabGard® ES, Class II, Type A2, Biological Safety Cabinet (NuAire, Plymouth, MN)
3. Benchtop Centrifuge with swinging bucket rotor (Beckman Coulter, model: Allegra X-14R)
4. Centrifuge (Thermo Fisher Scientific, model: Sorvall ST 16R)
5. CO₂ Incubator (Thermo Fisher Scientific, model: Heracell VIOS 160i)
6. Amnis ImageStream Mark II imaging flow cytometer (Luminex Corporation, Austin, TX, USA)

Software

1. Amnis INSPIRE (Luminex Corporation, Austin, TX, USA) for data acquisition
2. Amnis IDEAS (Luminex Corporation, Austin, TX, USA) for data analysis, available for download on the company's website

Procedure

A. Neutrophil isolation using Polymorphprep medium

Notes:

- a. Choose a high yield one step neutrophil purification method as this NETs detection protocol requires relatively high number of neutrophils (2×10^6 per sample) with minimal background activation.
- b. The centrifugation step must be performed in a swing bucket rotor as the $500 \times g$ speed has to be reached in the middle of the tube, at the interface between the blood and the Polymorphprep medium.
- c. It is not uncommon that the Polymorphprep separation fails when using blood from female donors. In our experience this separation works consistently with donors of both sexes that have a MHCH (mean corpuscular hemoglobin concentration) value within the normal range.
- d. Keep the purified neutrophils on ice at all times and use them for experiments within 2 h.

1. Collect whole blood in 10 ml EDTA (purple top) vacutainers and start neutrophil isolation within 1 h of the blood draw.

2. Equilibrate rotors and adaptors of the centrifuge to room temperature (18°C to 22°C). Set “Acceleration” to one; set “Deceleration” to zero. Set centrifugation to $500 \times g$ for 30 min.
3. Layer, very gently, undiluted whole blood on to the Polymorphprep medium always kept at room temperature, at 1:1 ratio and proceed to centrifugation. Centrifuged tubes will have the following layers from top to bottom: plasma, peripheral blood mononuclear cells – polymorphonuclear cells (neutrophils) – red blood cells (as shown in Figure 1B).
4. Collect the neutrophil ring and gently re-suspend in 50 ml ice-cold DPBS. Centrifuge at $400 \times g$ for 10 min at 4°C, with acceleration and deceleration set at maximum. Remove and discard platelets-containing supernatant.
5. Flick the pellet to resuspend, then add 3 ml ice-cold sterile water and make sure the pellet is fully re-suspended by gently pipetting up-and-down a few times. After 30 s restore osmolarity with 6 ml sterile ice-cold 0.6 M KCl. Fill up with ice-cold DPBS and centrifuge at $330 \times g$, 7 min at 4°C.
6. Discard the supernatant and re-suspend the pellet in complete neutrophil medium equilibrated at room temperature (RPMI supplemented with 2 mM L-glutamine) at 4×10^6 cells/ml.

B. Induction of NETosis

Notes:

- a. Adapt the choice of NETs inducers and the length of the treatment according to the experimental requirements.
 - b. Distinctive NETs agonists induce optimal responses at different lengths of the treatment period. Five time points (2, 7, 15, 30 and 60 min) and 5 different agonists (calcium ionophore, Hemin, LPS, IL-8 and PMA) have been tested in this protocol.
 - c. Allow purified neutrophils to rest before treatment with the NETs inducers to reduce their background activation.
 - d. For each experiment prepare fresh 8% PFA dilution in 1× DPBS and keep it cold and in the dark until needed (this will be further diluted to a final concentration of 4% in a further step. Do not store for second use).
 - e. All steps below can be performed directly in 1.5 ml Eppendorf tubes if so chosen. However, vigorously mixing the PFA into the cell suspension by pipetting can result in splashing and loss of cells. Using 5 ml tubes provides more space for thorough mixing and to prevent loss of cells.
1. Add purified neutrophils, 2×10^6 cells (in 500 µL of complete neutrophil medium) in a 5-mL sterile tube and allow the purified neutrophils to equilibrate at room temperature.
 2. Transfer tube to a 37°C incubator at 5% CO₂ and allow them to rest for at least 30 min without closing the cap.
 3. Add 100 µL of complete RPMI for the untreated control or the RPMI containing the NETs inducers of choice, mix gently the same number of times in all tubes and return tubes to incubator for the desired periods of time. Do not close the caps of the tubes during this incubation period.
 4. Remove from incubator and stop reaction by adding 600 µL of freshly made 8% PFA in 1× DPBS for a final concentration of 4% PFA, mix at least 5 times by pipetting, and allow to stand at least 30 min at room temperature, cover with aluminum foil.
 5. Add 1ml Wash Buffer (WB) at room temperature and centrifuge at $1,120 \times g$ for 5 min, at 4°C. Discard the PFA-containing supernatant into the designated chemical waste.

Note: To ensure that the PFA is removed, repeat this wash step one more time.

6. Gently re-suspended the pellet with 500 µL of WB and transfer it into 1.5 mL Eppendorf tube for subsequent staining steps.
7. Repeat the washing of the 5-mL tube with another 500 µL of WB to make sure all cells have been collected for staining.

C. NETosis staining technique for imaging flow cytometry

Notes:

- a. Remove the supernatant completely from cells at all steps, while paying attention not to lose cells in the process.
 - b. Determine the optimal working dilutions for all the antibodies used in the staining panel by titrating with a range of dilutions above and below of those recommended by the vendors (for example, for a recommended dilution of 1:1,000, up to 5 different dilutions should be tested – 1:250; 1:500, 1:1,000, 1:2,000, 1:3,000). This is required as the lasers on different ImageStream cytometers might have different powers.
 - c. Optimal H4cit3 dilution is defined as the concentration that provides the greatest separation of the positive staining against the background (and has highest index score when antibody titration is performed). Because the concentration of the antibody stock provided by the company might vary between lots, the optimal H4cit4 dilution has to be determined for each new lot of antibody.
 - d. Acquire the samples within 72 h of the end of the staining protocol. Longer wait times might interfere with the strength of the signal, particularly for Hoechst (which leaks freely from the stained cells with time). Our recommendation is to acquire them immediately.
 - e. Store stained cells at 4°C in the dark and keep them on ice and in the dark during acquisition on the ImageStream cytometer.
1. In the 1.5 mL Eppendorf tubes re-suspend fixed and thoroughly washed cells in 100 µL of 2% BSA containing CD66b-PE, mix vigorously at least 5 times by pipetting and incubate for 20 min, at room temperature, in the dark.
 2. Washing step: Add 500 µL WB and centrifuge at 1,120 × g for 5 min, at 4°C. Remove the supernatant and repeat washing two more times.
 3. Re-suspend washed pellet in 300 µL of BD Cytofix/Cytoperm, mix by pipetting up and down at least 5 times, incubate 15 min at room temperature, in the dark.
 4. Washing (step #2).
 5. Re-suspend washed pellet in 500 µL of blocking buffer, mix by pipetting up and down at least 5 times, incubate overnight at 4°C, in the dark.
 6. Washing (step #2).
 7. Re-suspend in 100 µL of 2% BSA containing anti-histone H4 citrulline 3 (H4cit3) primary antibody at the optimal determined dilution. Pipet up and down at least 10 times and incubate overnight at 4°C.

Note: The concentration of this primary antibody is the limiting factor for the final strength of the fluorescence signal (see note b above).

8. Washing (step #2).
9. Re-suspend pellet in 100 µL of 2% BSA containing secondary antibody DyLight 680, at the determined optimal dilution and the anti-MPO-AlexaFluor 594 conjugated. Pipet up and down at least 10 times and incubate for 30 min, at room temperature, in the dark.
10. Washing (step #2).
11. Re-suspend in 200 µL of 2% BSA containing Hoechst diluted as suggested by the vendor (1:1,000). Mix thoroughly by pipetting and incubate for 15 min, at room temperature, in the dark.
12. Add 500 µL WB and centrifuge at 1,120 × g for 5 min, at 4°C. Remove supernatant.
13. Re-suspend in 50 µL WB for ImageStream (Imaging Flow cytometer) acquisition (see notes d and e above).

D. Imaging flow cytometry acquisition

Within the INSPIRE software, establish the acquisition settings and create a template to be used with all experimental repeats.

1. Under the “Illumination” tab turn on the 785 laser (side scatter laser – SSC) and all other lasers to be used in the experiment, based on the fluorochromes panel (a chart showing the laser excitation wavelength and its corresponding dyes is available in the company’s website).
2. Under the “Magnification” section, adjust magnification to 60 \times .
3. Under the “Fluidics” section, slide the Speed/Sensitivity bar to the left (to ensure acquisition with low speed and high sensitivity).
4. Under “File Acquisition”, create a new folder to save the acquired files (with a .rif extension – *raw image file*).
5. Load a sample stained with all the colors and expected to yield the brightest signals for all the dyes used (stimulated with PMA as an example – the rational here is to ensure the positive fluorescent signal is within range and will not be saturating the channel).
6. Adjust the laser voltages based on the maximum of fluorescence without channel saturation of each fluorochrome according to their correspondent laser excitation.

Note: When setting the channel-specific laser power, the optimal voltage of the laser is the one that does not saturate the detectors (less than 4096 pixels in the generated image) and also promotes a clear distinction between negative and positive population. A ‘Raw Max Pixel’ feature of each of the used channels should be applied. This feature provides the highest value of the pixels contained in the input mask.

7. Once the laser voltages are set, “Return” the sample and set it aside to re-acquire it after the single color compensation control tubes have been acquired for spillover matrix calculation.
8. To generate a compensation matrix file, start the compensation wizard on Inspire and acquire at least 1,000 events from each of the compensation control tubes.

Note: The customers portal on the Amnis ImageStream website provides detailed instructions of how to run and validate compensation matrix using the Wizard in the INSPIRE (acquisition software). A compensation matrix has to be applied to every acquired .rif file when first opened for analysis in the IDEAS software (even when a compensation matrix was also applied during the acquisition of that file with INSPIRE). Spillover compensation can be done during acquisition or post-acquisition of data files. If the stop gates on acquisition of the files are based on fluorescent signals, for example, acquisition based on 5,000 positive cells for CD66b, then it is advisable to perform compensation on INSPIRE (use the compensation wizard) to ensure that the fluorescent signal is corrected for spillover in other fluorescent channels.

9. Create a dot plot of Area of the Brightfield (BF) versus intensity of Side Scatter (SSC) channels and by clicking around the dots (clusters of populations) and evaluating the correspondent imagery displayed in the gallery, the single cells can be found and boundaries determined. In this protocol, for purified neutrophils, the gate of single cells should be in the range of 50 μm^2 to 200 μm^2 area. This should exclude most of the debris, small particles (to the bottom and left) and aggregates (slightly upper and right – please refer to Figure 2A).

Note: Due to the nature of neutrophils, we thought it would be easier to identify singlets by the use of Area versus SSC; however, one can also use Area versus Aspect Ratio (AR) of BF to determine single cells. Aspect Ratio is the ratio of width by the height, events closer to AR of 1 will be where the single events should be located.

10. From the gate above, create a histogram using the gradient RMS (root mean square) feature of the BF (Channel 1 or 9), to select the cells in focus. Because this feature measures the sharpness quality of an image, changes of pixel values of 40 and above, usually corresponds to the cells in best focus. Selecting the bin and visualizing the correspondent cells within the bin in the image gallery will help determine where to begin the focused gate in this histogram.

11. Set the “Acquisition” tab to acquire 20,000 single and focused cells (first and second plots created, respectively).
12. Save this template to be used for subsequent acquisitions with the same settings for laser powers, magnification and number of events acquired.
13. Type the name of the sample into the “Acquisition” tab.
14. “Load” the sample, confirm cells are centered and in focus and click “Acquire”.

Note: Follow one of the two available brightfield channels (channel 1 or channel 9) to confirm the cells are centered (i.e., the cells are flowing in the middle of the channel) and in focus (the images of the flowing cells are clear and crisp).

Data analysis

Notes:

- a. Representative images for steps 3 to 5 are shown in Figure 2A.
- b. For a full set of experimental results with our tested NETs agonist refer to our previous publication (Barbu et al., 2020).

1. In the IDEAS analysis software, open the .rif extension file to be analyzed.
2. Apply the compensation matrix to be used.

Note: A previously made compensation matrix is stored as a .ctm file. IDEAS software will prompt for selection of a compensation matrix for every .rif file that needs to be analyzed. Navigate to the stored file and load it by using the instructions from the analysis software.

3. Create a dot plot of area of the BF image (M01 – default mask) versus side scatter (SSC – channel 6).

Note: Single neutrophils with high SSC and relatively big cellular area will appear as a distinct clustered population. Check a few of the single cells to define the boundaries of the singlets gate and exclude debris and mononuclear cells. The best way to determine and validate the gate boundaries, besides the visible cluster of events in the graph, is to click on some dots in the plot and visualize the correspondent imagery. When looking on Area axis, one should expect single events to the right of the population of debris or smaller cells and to the left of doublets, larger aggregates or even bigger cells, if the cell suspension may contain such cells.

4. Select the focused cells by using the focus quality feature in brightfield, Gradient RMS (Root Mean Square).
5. Create a dot plot of signal intensity for granulocyte-specific marker CD66b versus Hoechst and gate double positive events (CD66b⁺Hoechst⁺) for further analysis.
6. In the CD66b⁺Hoechst⁺ gate create a histogram for the intensity of the H4cit3 signal and evaluate percent of H4cit3⁺ cells and/or mean/median intensity fluorescence for this marker (Figure 2B).

Note: We recommend the use of median intensity fluorescence for this parameter.

7. On the nuclear channel (Hoechst) the CD66b⁺Hoechst⁺ gate create a dot plot of ‘Area’ versus the specific texture feature ‘Bright Detail Intensity_R3’ (BDI_R3) to identify neutrophils with decondensed nuclei from those with normal nuclei in the CD66b⁺Hoechst⁺ gate (Figure 2C).

Note: BDI is a feature that calculates intensity of localized bright spots within the masked area in the image (the description of this and other IDEAS features used in this protocol can be found in the downloadable IDEAS ImageStream Analysis Software User’s Manual).

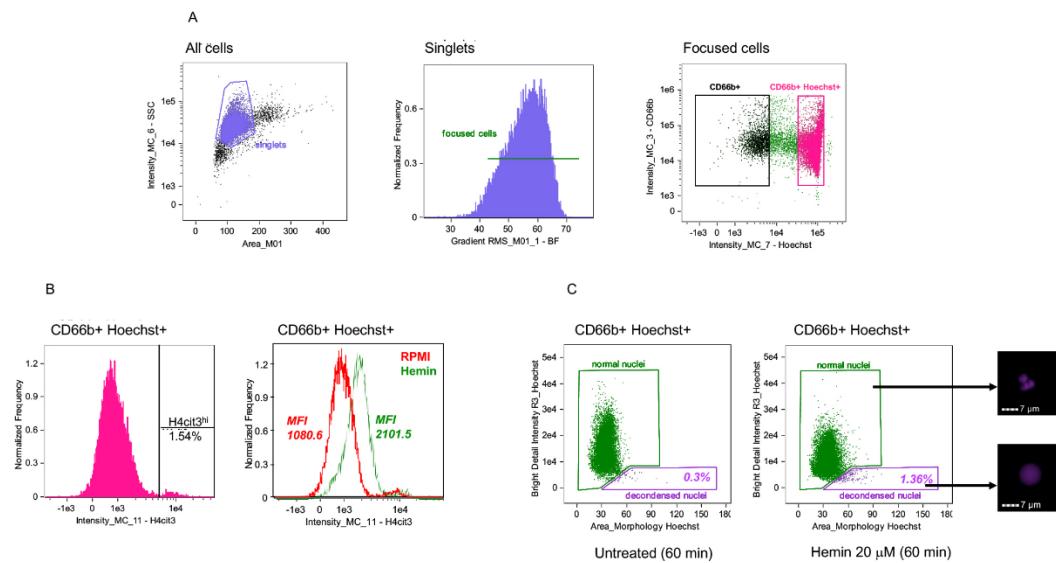


Figure 2. Gating strategy for identification of neutrophils and their nuclear NETosis markers by using IDEAS software.

A. Single cells identified with two-parameter dot-plot of area of Brightfield (BF – X axis) versus Intensity of Side Scatter on Channel 6 (SSC – Y axis) (left panel). Focused singlets cells selected by using Gradient RMS (Root Mean Square) in Brightfield (middle panel). Neutrophils ($CD66b^+Hoechst^+$, in strawberry red) identified from singlet focused cells in a two-parameter dot-plot of fluorescence of Hoechst on Channel 7 (X axis) and $CD66b$ on Channel 3 (Y axis) (right panel). **B.** Representative histograms showing the percentage of $H4cit3^{hi}$ cells in the $CD66b^+Hoechst^+$ gate and $H4cit3$ median fluorescence intensity (MFI). In the right panel the neutrophils were left untreated in RPMI (red) or were treated with 20 μ M Hemin for 15 min (green). **C.** Decondensed nuclei (purple) and normal nuclei (green) identified with a dot-plot of area of nucleus (morphology mask function of Hoechst staining on Channel 7) versus Bright Detail Intensity R3 of the Hoechst staining (examples of nuclei from the two gates are included on the right side of the panel). Neutrophils were left untreated (left) or were treated with 20 μ M Hemin for 60 min (right). Percentage of cells with decondensed nuclei is shown. Scale bars = 7 μ m.

8. Use Similarity feature on the nuclear dye channel (Hoechst) and MPO channel to determine the degree to which the two images are linearly correlated within the masked nuclear area in cells with normal and decondensed nuclei (Figure 3A).

Note: When located in the cytoplasm MPO staining has a dissimilar distribution compared to the Hoechst-stained nuclear mask (i.e., low Similarity score, no co-localization). When the intensity of both dyes at the nuclear location is high, the high Similarity values indicate co-localization.

9. Use the customized “Level Set” mask on the nuclear dye channel and the ‘LobeCount’ feature to detect reduction in the percentage of cells with multi-lobulated nuclei and separately quantify cells with variable number of lobes (1-lobe to 4-lobes) (Figure 3B).
10. In the 1-lobe gate create a dot plot of ‘Area of nucleus’ versus ‘Ratio of nuclear by the whole cell areas’ to quantify nuclear supercondensation. Supercondensed nuclei have low values for both parameters; (Figure 3C).

Note: See the stand-alone Notes chapter for instructions on how to construct this custom feature. For better resolution of the nuclear area, custom masks should be used for the above-mentioned parameters.

- In the normal nuclei gate construct a dot plot of ‘Standard deviation’ (X axis) and ‘Modulation’ (Y axis) to assess changes in cell membrane texture. High values for Standard deviation in darkfield (SSC channel) and for Modulation in brightfield indicate neutrophils with damaged membranes (Figure 3D).

Note: The Standard Deviation Feature provides information on the complexity of an object. Modulation Feature can quantify image quality and thus characterize cells' texture.

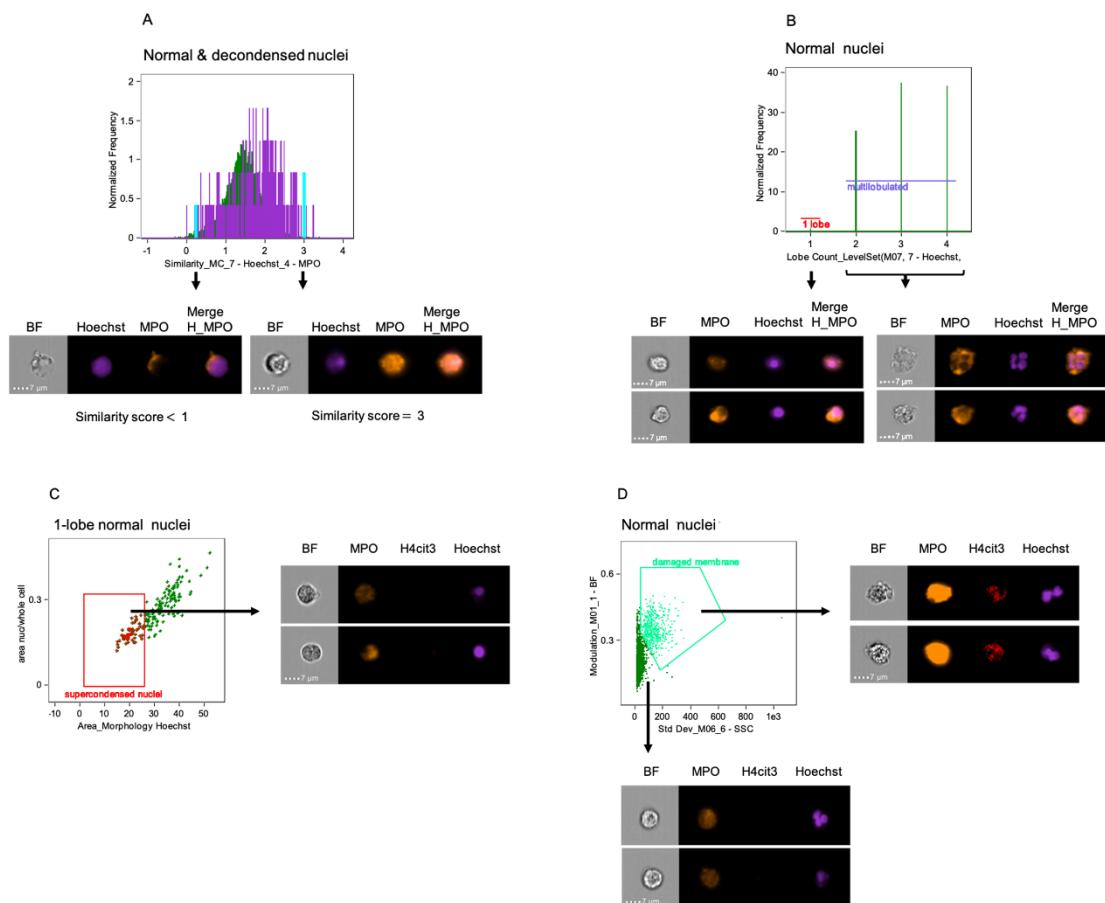


Figure 3. IDEAS software analysis features to identify and quantify additional nuclear and cellular alterations.

A. Similarity analysis feature for DNA (Hoechst – channel 7) and MPO (AF-594 – channel 4) indicates co-localization of the nuclear compartment with type I granules in neutrophils with normal nuclei (green) and decondensed nuclei (purple). Examples of cells with low (< 1) and high (3) similarity scores, indicating low and high DNA-MPO co-localization, respectively, are included (the position of their specific bins on the graph is highlighted in aqua). **B.** Lobe Count feature identifies changes in the number of nuclear lobes based on nuclear imagery and quantifies the number of cells in each category. Representative images of neutrophils with 1 lobe or multilobed nuclei are included. **C.** Dot-plot of Area of Nucleus (morphology mask of Hoechst staining) on X axis versus ratio of the nucleus by the whole cell areas in the 1 lobe nucleus gate identifies supercondensed nuclei with low nuclear area and consequently low ratio when divided by the whole cell area. Representative images of cells with supercondensed nuclei are presented. **D.** Neutrophils with damaged membranes (bright green) are distinguished with two-parameter dot-plot of Standard Deviation Side Scatter on Channel 6 (X axis) versus Modulation of Brightfield (Y axis). High scores for both features indicate complex and textured objects. Included, representative imagery of cells within both gates. (BF = Brightfield; H = Hoechst). Scale bars = 7 μ m.

12. Save file once all the analysis parameters have been established. For each .rif acquisition file, two additional files (.daf and .cif) are created in the analysis step. Use the .daf for analysis. Do not remove the .cif one (this is where all analysis data is stored).
13. Use “Batch Data File” under the “Tools” tab to assign all saved analysis parameters to the remaining .rif file to be analyzed.
14. In the .daf analysis file use “Reports” to define a statistic report for all parameters of interest in the target subpopulations and then “Generate Statistic Report” to apply it to all analyzed sample files (Figure 4).

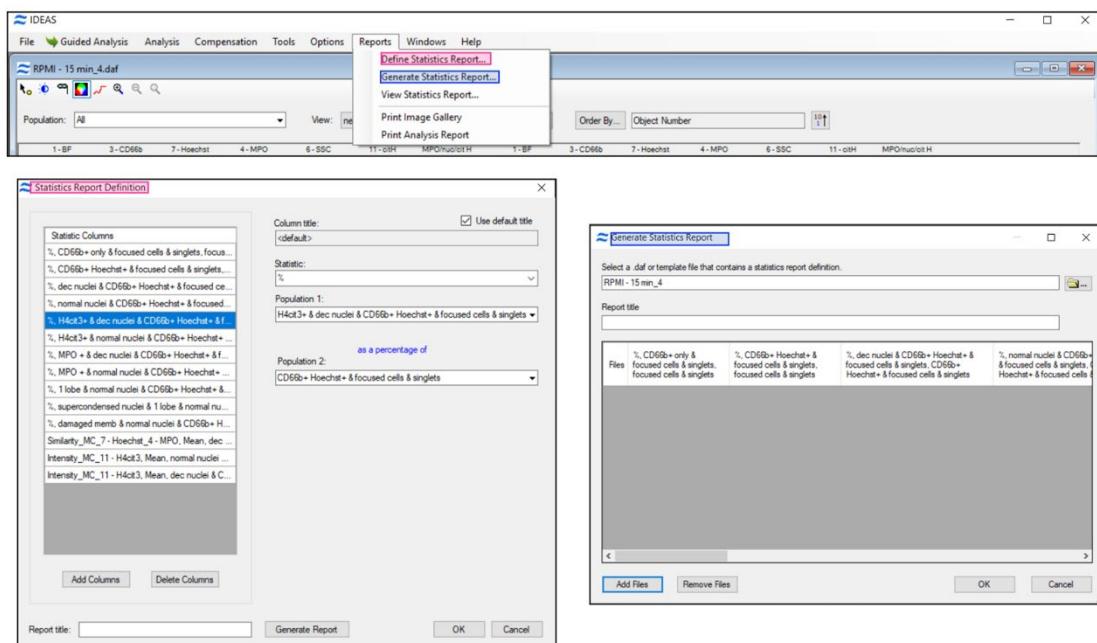


Figure 4. Define and generate a statistic report with all analysis parameters of interest.

Use “Add Files” function to apply all defined stats to the .daf analysis files. IDEAS software generates and stores a .txt statistics file that can be opened with Excel for further processing.

Notes

1. In this protocol all steps (neutrophils isolation and stimulation, staining, detection and NETs quantification) can critically impact the data quality.
2. Neutrophils are notorious for being easily activated during purification. Rest the purified population for 30 min in the incubator prior to the NETs stimulation to reduce false positive data.
3. Confirm purification of neutrophil population by additional methods (*e.g.*, cytopspin).
4. Stop the NETs challenge step by adding PFA into the reaction mix, rather than spinning the tube and removing the supernatant before adding the PFA to reduce activation by other means.
5. Confirm the lack of non-specific binding for the secondary antibody (DyLight 680) by running a control tube with cells and secondary DyLight 680 antibody but no H4cit3 primary antibody.
6. Use custom masks for the data analysis with the IDEAS software, as they represent more accurately the region of interest. Validate the custom masks for total cell area and nucleus, by confirming that the masks are indeed masking the region(s) of interest (*i.e.*, not missing parts of the target region or, on the opposite, masking ‘ghost’ regions outside of what is relevant for the features) (Dominical *et al.*, 2017).
7. Note that this protocol allows the identification of NETosis markers in whole neutrophils, prior to the release of the DNA and the cytoplasmic proteins strings into the extracellular space. This detail is crucial for deciding the length of the challenge with the NETs inducers for a peak number of cells that respond to

stimulus and can be acquired with the ImageStream cytometer. Lengthy stimulation periods might lead to false negative results due to the fact that the cells have already lysed and released their content and thus are no longer “visible” to the flow cytometer. This case can be confirmed by independent assessments: for example, by the size of the pellet observed in the staining tube, and the number of events in the CD66b⁺Hoechst⁺ gate actively acquired by the flow cytometer in the stimulated tube being reduced as compared to the untreated control.

8. To generate a custom feature to evaluate nuclear supercondensation:
 - a. In the IDEAS software, open a .daf extension file, select a Hoechst⁺CD66b⁺ double positive cells.
 - b. On the top menu in IDEAS, select ‘Analysis’, then ‘Masks’. In the open pop-up window select ‘New’, then ‘Function’.
 - c. To create a mask for the nuclear staining: select the fluorescent channel for the nuclear imagery for both the image and the channel boxes. In the drop-down menu of the function masks, test which mask function fits best with the nuclear image by looking at the changes of masking in the imaging box (usually “Morphology” works best as nuclear mask, as it covers more accurately the nuclear stained region) (Dominical *et al.*, 2017). Once the new function mask is chosen, Click ‘Ok’.
 - d. Create a name for that mask or use the default name suggested by the software.
 - e. Click ‘Ok’ again to generate the mask.
 - f. To create a new mask press ‘New’.
 - g. Repeat steps b through e to mask the whole cell area by using CD66b imagery or BF channel. “Object” or “Erode” function masks are good choices for masking the cell region.
 - h. Click ‘Close’ to close the mask window.
 - i. To apply new features on these new custom masks created, go back to ‘Analysis’ in the top menu bar and select ‘Features’. Select “New” in the pop-up window.
 - j. ‘Feature Type’ box is available for choosing an option. Choose ‘Single’ and in the drop-down menu in the side, choose the ‘Area’ feature.
 - k. Below the ‘Feature Type’ box, in the mask box, select the nuclear mask newly created. ‘Set a Default Name’ or create a new name. Click ‘Ok’ when done.
 - l. Repeat steps i to k. Next use the ‘whole cell’ mask generated to input in the mask box of the new area feature to create the new ‘Area of the whole cell’ feature.
 - m. To calculate the ratio of these new features created, make a combined feature by selecting ‘New’ in that same feature window.
 - n. In the ‘Feature Type’ box select ‘Combined’.
 - o. In the ‘Features Box’ on the left, select the ‘nuclear area’ feature created.
 - p. Highlight the feature and on the right side of the window select the ‘arrow down’ to insert that feature in the box.
 - q. Select “/” (forward slash) to calculate the ratio.
 - r. Highlight the ‘Area of the whole cell’ new feature created and press the arrow down to insert this feature in the box after the ratio sign. The nuclear area is now divided by the whole cell area.
 - s. Name the new feature. Click ‘Ok’ when done.
 - t. Click ‘Close’ to close the Features window. This new feature can be now applied in histograms or two-parameters dot plot.

Recipes

1. 8% Paraformaldehyde (PFA) working solution
2.5 mL PFA 32% stock
Add 10 mL 1× DPBS, no calcium, no magnesium
2. Wash Buffer (WB)
10 g BSA (final concentration 2%)
2 mL EDTA from 0.5 M stock (final concentration 2 mM)
Add 500 mL 1× DPBS, no calcium, no magnesium

- Filter through 0.45 µm pores
3. Blocking Buffer (BB) (keep sterile)
15 g BSA (final concentration 3%)
5 ml porcine skin gelatin stock (final concentration 0.2%)
Add 50 mL 1× DPBS, no calcium, no magnesium
Filter through 0.45 µm pores
4. Porcine skin gelatin stock 2%
2 g porcine skin gelatin
100 mL ddH₂O
5. 0.6 M KCl (keep ice-cold)
37.28 g KCl granular
1,000 mL ddH₂O
Filter through 0.45 µm pores
6. Neutrophils complete medium (keep sterile)
5 mL L-glutamine from 200 mM stock (final concentration 2 mM)
500 mL RPMI-1640 without L-glutamine

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Competing interests

The authors declare no competing financial or non-financial interests.

Ethics

Informed consent was obtained from all subjects who provided blood samples enrolled in the study protocol NCT0004799, approved by the NHLBI Institutional Review Board.

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Assay for Assessing Mucin Binding to Bacteria and Bacterial Proteins

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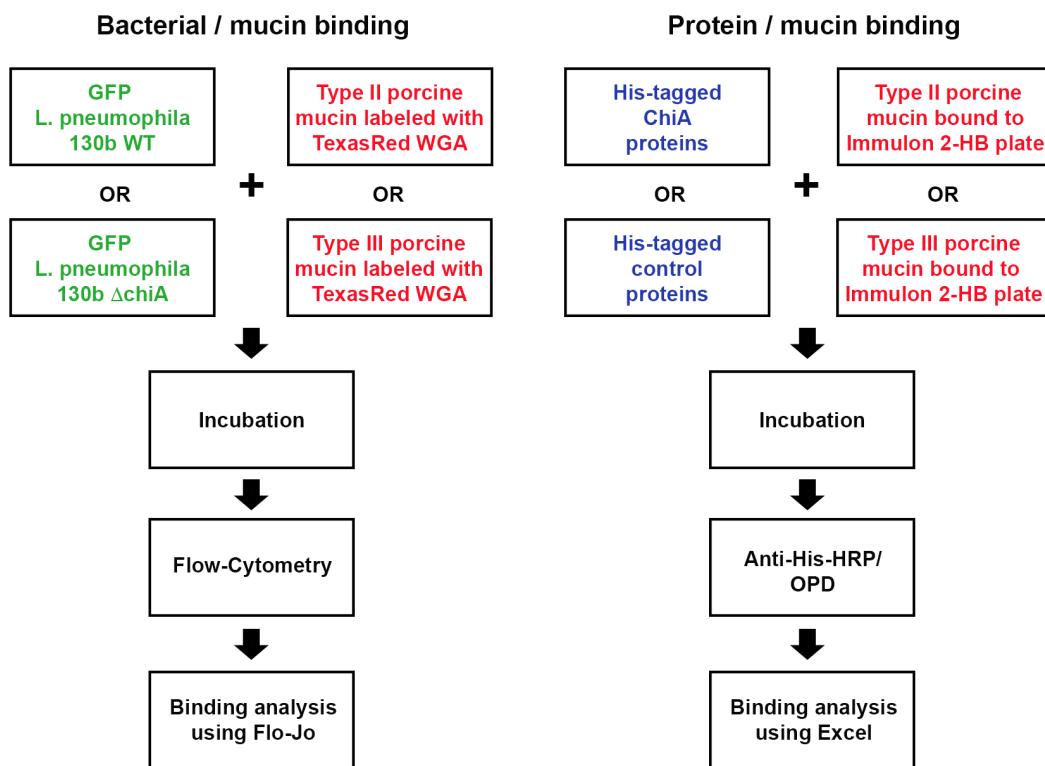
Abstract

Legionella pneumophila, a Gram-negative bacterium and the causative agent of Legionnaires' disease, exports over 300 effector proteins/virulence factors, through its type II (T2SS) and type IV secretion systems (T4SS). One such T2SS virulence factor, ChiA, not only functions as a chitinase, but also as a novel mucinase, which we believe aids ChiA-dependent virulence during lung infection. Previously published protocols manipulated wild-type *L. pneumophila* strain 130b and its *chiA* mutant to express plasmid-encoded GFP. Similarly, earlier studies demonstrated that wheat germ agglutinin (WGA) can be fluorescently labeled and can bind to mucins. In the current protocol, GFP-labeled bacteria were incubated with type II and type III porcine stomach mucins, which were then labeled with TexasRed-tagged WGA and analyzed by flow-cytometry to measure the binding of bacteria to mucins in the presence or absence of endogenous ChiA. In addition, we analysed binding of purified ChiA to type II and type III porcine stomach mucins. This protocol couples both bacterial and direct protein binding to mucins and is the first to measure Gram-negative bacterial binding to mucins using WGA and flow-cytometric analysis.

Keywords: Mucin-bacteria binding, Mucin, ELISA, Mucin-binding proteins, Bacterial flow-cytometry, *Legionella pneumophila*

This protocol was validated in: PLoS Pathog (2020), DOI: 10.1371/journal.ppat.1008342

Graphical Abstract:



Strategy for assessing bacterial and protein binding to mucins.

Background

Legionella pneumophila (*Lpn*), a Gram-negative bacterium, is the causative agent of Legionnaires' disease, a severe form of pneumonia. *Lpn* is an intracellular pathogen that produces over 300 protein effectors that it secretes through either a type II secretion system (T2SS), or through a type IV secretion system (T4SS) (Hubber and Roy, 2010; White and Cianciotto, 2019). ChiA is one such T2SS protein effector. ChiA is an 81-kDa endochitinase that has a role in *Lpn* virulence during lung infection (Rehman *et al.*, 2020). *Lpn* carrying a deletion of the ChiA gene (*ΔchiA*) shows decreased survival in the lungs of mice, compared to WT *Lpn* (DebRoy *et al.*, 2006). While humans do not produce chitin, they do produce analogous glycoproteins, mucins, that have known properties in interacting with and blocking infection of other pathogens. In our study, we showed, for the first time, that ChiA is able to both bind to and degrade mucins (Rehman *et al.*, 2020). To determine if live bacteria were able to bind to mucins, we utilized our previously published protocol to manipulate wild-type *Lpn* strain 130b and its *chiA* mutant to express a GFP-producing plasmid (DebRoy *et al.*, 2006; Rondelet and Condemeine, 2013). Furthermore, earlier studies showed that wheat germ agglutinin (WGA) binds to mucins (Bhavanandan and Katlic, 1979; Valdizan *et al.*, 1992). Therefore, we utilized porcine stomach mucins which we labeled with TexasRed-conjugated WGA (Model *et al.*, 2009). To determine whether ChiA directly binds to these mucins, we also used an ELISA based-assay with purified recombinant (N-terminally His-tagged) ChiA and detected binding using anti-His₆ antibodies.

Although mucin binding to bacteria has been studied previously (Naughton *et al.*, 2014), and fluorescently labeled WGA has been studied in the context of Gram-positive bacteria (Fife *et al.*, 2000) this is the first protocol to directly label mucins and bacteria and then utilize flow cytometry to measure mucin binding to a Gram-negative bacterium. Furthermore, by analyzing both bacterial binding to mucins and the binding of purified proteins to mucins, this protocol provides insight into synergistic binding of different surface exposed bacterial proteins to mucins. This

protocol can be further applied to study mucin binding to other Gram-negative bacteria.

Part I: Bacterial Mucin Binding with Flow Cytometry

Materials and Reagents

1. *Legionella pneumophila* Brenner *et al.* (1979) (ATCC® BAA-74™)
 - a. WT 130b purchased from ATCC (see above)
 - b. ChiA mutant as previously reported (DebRoy *et al.*, 2006)
 - c. GFP plasmid (pMGFP), as previously reported, was derived from pMMB-GRN/pMMB-Gent (addgene 45475). GFP is expressed from a Ptac promotor and therefore IPTG is required (White and Cianciotto, 2016) (Sturgill-Koszycki and Swanson, 2000). While copy number in *Legionella* is unknown, the PMMB-Gent derives from PMMB67EH (ATCC 37622)
2. 1× Phosphate Buffered Saline (PBS) (Corning, catalog number: 21-040-CM)
3. IPTG (Sigma-Aldrich, catalog number: I6758)
4. Type II porcine stomach mucin (Sigma-Aldrich, catalog number: M1778)
5. Type III porcine stomach mucin (Sigma-Aldrich, catalog number: M2378)
6. TexasRed-tagged WGA (ThermoFisher Scientific, catalog number: W21405)
7. Sodium carbonate, Na₂CO₃ (Sigma-Aldrich, catalog number: S7795)
8. Sodium bicarbonate, NaHCO₃ (Sigma-Aldrich, catalog number: S5761)
9. Buffered Charcoal Yeast Extract (BCYE) agar plates (see Recipes)
 - a. ACES (Sigma-Aldrich, catalog number: A9758)
 - b. KOH (Sigma-Aldrich, catalog number: 221473)
 - c. Yeast Extract (Sigma-Aldrich, catalog number: Y1625)
 - d. α-Ketoglutaric acid sodium salt (Sigma-Aldrich, catalog number: K1875)
 - e. Activated Charcoal (Sigma-Aldrich, catalog number: C9157)
 - f. Bacteriological Agar (Sigma-Aldrich, catalog number: A5306)
 - g. L-cysteine HCl (Sigma-Aldrich, catalog number: C1276)
 - h. Ferric pyrophosphate (Sigma-Aldrich, catalog number: P6526)
10. 50 mM Carb/Bicarb Buffer (see Recipes)
11. Mucin solution (see Recipes)

Equipment

1. Flow Cytometer (<https://www.bdbiosciences.com/en-us/go-campaign/lsr-ii-comp-cont>) using Blue Laser (488 nm), Long pass Filter 600 and 505, Band Pass Filter 600-620 and 500-550 (BD Biosciences, model: LSR II)
2. General Purpose UV/Vis Spectrophotometer (Beckman Coulter, model: DU720)
3. Forced Air Microbiological Incubators (VWR, catalog number: 89511-430)

Software

1. GraphPad Prism version 8.0.0 for Mac (GraphPad Software, San Diego, California USA, www.graphpad.com)
2. FlowJo™ Software for Mac, Version 8.8.6. (Ashland, OR: Becton, Dickinson and Company, www.flowjo.com)

Procedure

A. *Lpn* Preparation

1. Streak out *Lpn* strain 130b and ChiA mutant NU319, both harboring a GFP-expressing plasmid, onto BCYE agar plates containing IPTG at 1 mM.
2. Incubate the plates for 3 days in a 37°C incubator to grow out a lawn of bacteria.
3. Resuspend ~3 swabs of scraped bacteria into 5 mL of PBS and read the optical density (OD) of each suspension at 660 nm using a DU720 spectrophotometer. Use PBS to dilute the bacterial suspension to an OD of 0.3, which corresponds to approx. 1×10^9 CFU/ml.

B. Mucin Preparation

Prepare both type II and type III porcine stomach mucin solutions in carb/bicarb buffer according to the recipe below.

C. Mucin/*Lpn* Incubation

1. Statically incubate 1 mL of the *Lpn* suspension with 100 µL of either type II porcine stomach mucin, type III porcine stomach mucins, or PBS for 1 h in a 25°C or 37°C incubator. Repeat every condition in triplicate.

Note: Keep an aliquot of the type II and type III porcine stomach mucin solutions (no bacteria) as controls for the flow-cytometry analysis.

2. Centrifuge, at room temperature, the mixtures for 5 min at 4,000 × g (for the 37°C samples) or 8,000 × g (for the 25°C samples).
3. Wash each pellet in 1 mL of PBS
4. Repeat centrifugation and wash step (Steps C2 and C3) three times.
5. Resuspend pellet in 1 mL of PBS containing 7.5 µg of TexasRed-tagged WGA and incubate statically for 15 min at 25°C.
6. Repeat the centrifugation and wash steps (Steps C2 and C3) three times.
7. Resuspend the pellet in 500 µL of PBS.

D. Flow Cytometry

Use BD LSRII flow cytometer to analyze suspensions via a TexasRed filter (Blue Laser – 488 nm: Long Pass 600, Band Pass 600-620) and GFP Filter (Blue Laser – 488 nm: Long Pass 505, Band Pass 500-550).

1. Set gating TexasRed and GFP parameters using only mucin and only bacteria respectively.
2. Collect a minimum of 500,000 events.

Data analysis

The experiment was replicated a minimum of three times, and each experiment had three technical replicates. FlowJo software was used to analyze percent of mucin binding to *Lpn*. Bacteria capable of expressing GFP and isolated mucin were used to set thresholds for both the TexasRed- and GFP-negative populations (unlabeled mucins and no IPTG-dependent GFP expression) and the TexasRed- and GFP-positive populations (TexasRed labeled mucins and IPTG-induced GFP expression). After setting these thresholds (see below for the four histograms on the left), the percent population (see below for quadrant analysis in the right-most flow-plot) of single GFP-positive (Quadrant [Q]3), single TexasRed-positive (Q1), and double GFP and TexasRed positive (Q2) were calculated using

FloJo quadrant tool. Thus, Q2 was used as a calculation of percent of mucin binding to bacteria. Due to variability in mean fluorescence between experimental days, data were normalized to GFP-Lpn incubated with TexasRed fluorophore alone (background), see sample data table below and Figure 4D (Rehman *et al.*, 2020). GraphPad Prism8 statistical software was used to graph and analyze the percent binding of bacteria to mucins. Prism8 analysis quantified standard deviation and used the two-tailed Student's *t*-test function to test for significant differences between groups tested. Only *P*-values less than 0.05 were determined to be significant.

Flow Cytometry Protocol (Figure 1)

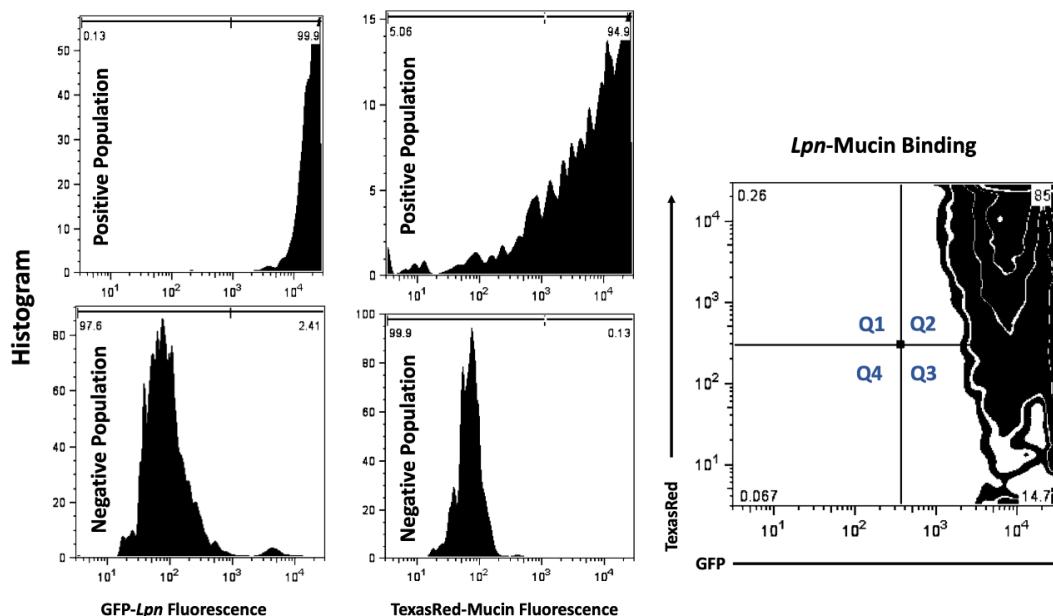


Figure 1. Flow Cytometry.

Thresholds were set using single-positive samples 1. IPTG induced GFP production in *Lpn* (top left histogram) and 2. non-GFP bacteria with TexasRed labeled mucins (top middle histogram). Single negative populations were set using 1. Bacteria cultured without IPTG (bottom left histogram) 2. Non-GFP bacteria with un-labeled mucins (bottom middle histogram). For sample analysis, IPTG induced, GFP-expressing bacteria were co-incubated with TexasRed-labeled mucins. Gating parameters were used to setup quadrant percentiles for analysis. All events collected were partitioned into four quadrants using FloJo tool. Percentage of total population that were only TexasRed positive were labeled Q1, double GFP and TexasRed bacterial populations were labeled Q2, single GFP-positive populations were designated Q3 and non-fluorescent, all negative, populations were designated Q4. Percent of population that was GFP and TexasRed double positive (Q2) was used as a metric for mucin-bacteria binding and was analyzed in the sample table (Table 1) below.

Sample Data (Table 1)

Table 1. Percent of population that was double positive for TexasRed-mucin and GFP-Lpn (Q2) was used as

	Rep1 GFP+ TexasRed+ Q2	Rep2 GFP+ TexasRed+ Q2	Rep3 GFP+ TexasRed+ Q2	Normalization	PBS Normalized Rep1	PBS Normalized Rep2	PBS Normalized Rep2	Mean	Stdev
WT_PBS_TexasRed	3	4	5	(GFP + TexasRed+ Quadrant 2)/(Quadrant 2 WT PBS)	3/3=1	5/5=1	6/6=1	1	0
ChiA_PBS_TexasRed	2	3	4	(GFP + TexasRed+ Quadrant 2)/(Quadrant 2 ChiA PBS)	5/5=1	6/6=1	7/7=1	1	0
WT_TexasRed_Mucin	85	87	88	(GFP + TexasRed+ Quadrant 2)/(Quadrant 2 WT PBS)	85/3=28.3	87/4=21.7	88/5=17.6	22.5	5.4
ChiA_TexasRed_Mucin	89	91	92	(GFP + TexasRed+ Quadrant 2)/(Quadrant 2 ChiA PBS)	89/2=44.5	85/3=28.3	92/4=23	31.9	11.2

a value for mucin binding to bacteria. In the single example of WT behavior in the flow plot shown above, this value was 85% (right-most plot in Figure 1). This value plus the values obtained from two additional independent replicates testing WT bacteria were added to the third row in the first three columns on the left, *i.e.*, under headers Rep1, Rep2, Rep3. The raw values for three technical replicates obtained from analysis of another bacterial sample; *i.e.*, a ChiA mutant, appear in the three left-most lanes in row-4. The value for each of the replicates was then normalized to GFP-bacteria (WT or mutant) incubated with TexasRed alone (as noted in rows 1 and 2), to quantify background TexasRed fluorophore binding to bacteria. For the two bacterial samples incubated with mucin, these values appear in columns 5, 6, and 7 for rows 3 and 4. Finally, the normalized values were averaged, and standard deviations calculated, as indicated in the last two columns. In this sample dataset, $22.5 \pm 5.4\%$ of WT bacteria significantly bound the mucin.

Recipes

1. BCYE agar plates

- a. Add 10 g of ACES to ~900 mL of double distilled water. Stir into solution
- b. Add ~2.2 g KOH. Stir into solution
- c. Add 10 g of Yeast Extract and 1 g α -ketoglutaric acid. Stir into solution
- d. Adjust the pH of the broth to 6.85-6.95. Use concentrated HCl or 10 N KOH as required. Add double distilled water to 1 L
- e. Dispense the broth into a 2-L flask
- f. Add 1.5 g activated charcoal and 15 g of agar
- g. Autoclave 20 min at 121°C
- h. Prepare cysteine and iron solutions respectively by adding 0.4 g of L-cysteine HCl to 10 mL double distilled water and 0.25 g ferric pyrophosphate to 10 mL double distilled water. Once each solution is made, sterilize the solution by filter (0.22 μ m).
- i. Cool the medium to 50°C in a water bath after autoclaving
- j. Add separately 10 mL filter-sterilized (0.22 μ m) cysteine and 10 mL filter-sterilized (0.22 μ m) ferric pyrophosphate to the medium
- k. If making plates with IPTG, add 1 mL of 1 M Filter Sterilized (0.22 μ m) IPTG
- l. Cool to 40°C
- m. Pour plates and store at 4°C once solidified

2. 50 mM Carb/Bicarb Buffer

- a. Dissolve 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ in 1 L of deionized water
- b. Adjust the pH to 9.6 using HCl or NaOH

3. Mucin solution

Stir for 30 min to dissolve 10 mg of either type II or type III porcine stomach mucin in 100 mL of 50 mM Carb/Bicarb buffer above. Autoclave for 15 min at 135°C to sterilize and to further dissolve the mucin*.

**Note: Mucin is difficult to dissolve and will not fully go into solution by mixing alone; thus, autoclaving is required to get a homogeneous solution. Fresh preparation of mucin is recommended for each experiment.*

Part II: Recombinant Protein Mucin Binding with ELISA

Materials and Reagents

1. Immulon 2-HB 96-well plates (VWR, catalog number: 735-0464)
2. Purified N-terminally His-tagged protein samples
3. Anti-His HRP-conjugated antibody (Sigma-Aldrich, catalog number: SAB4301134)
4. Mucins from porcine stomachs type II (Sigma-Aldrich, catalog number: M2378)
5. Mucins from porcine stomachs type III (Sigma-Aldrich, catalog number: M1778)
6. Sodium carbonate, Na₂CO₃ (Sigma-Aldrich, catalog number: S7795)
7. Sodium bicarbonate, NaHCO₃ (Sigma-Aldrich, catalog number: S5761)
8. Bovine serum albumin (BSA) (Melford, catalog number: A30075)
9. Phosphate buffered saline (PBS) (Fisher Scientific, catalog number: BP399-1)
10. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
11. Zinc chloride, ZnCl₂ (Sigma-Aldrich, catalog number: 229997)
12. Tween 20 (Sigma-Aldrich, catalog number: P1379)
13. o-Phenylenediamine dihydrochloride tablets (OPD) (Sigma-Aldrich, catalog number: P9187)
14. SnakeSkin® dialysis tubing (Thermo Scientific, catalog number: 68100)
15. Incubation buffer (see Recipes)
16. 50 mM Carb/Bicarb Buffer (see Recipes)
17. Mucin solution (see Recipes)
18. Blocking buffer (see Recipes)

Equipment

1. Plate reader (LabSystems iEMS Reader MF, catalog number: 5921200) using 450 nm filter
2. Incubator (New Brunswick Innova 4230)
3. Centrifuge (Eppendorf, model: 5810R)

Software

1. Ascent Microplate Reader Software version 2.7.0 for Windows
2. Microsoft Excel version 16.43 for Mac; although any database analysis software is appropriate

Procedure

A. Protein preparation

Dialyze sample proteins at 100 µM into incubation buffer, prepared according to the recipe below. Adjust the final protein concentration after dialysis to 10 µM.

B. Mucin preparation

Prepare both type II and type III porcine stomach mucin solutions in carb/bicarb buffer according to the recipe below.

C. Incubation

1. Statically incubate an Immulon 2-HB 96-well plate with 50 µL of either type II porcine stomach mucin, type III porcine stomach mucins, or PBS overnight in a 4°C incubator. Repeat every condition in triplicate.
2. Remove the mucin solution and block the wells with 200 µL of blocking buffer, according to the recipe

below, statically for 1 h in a 24°C incubator.

3. Remove the blocking buffer and wash once by adding 200 µL of incubation buffer and incubating statically on the bench for 5 min. Next incubate the wells statically for 3 h in a 24°C incubator with 50 µL of purified protein samples or PBS.
4. Wash with 200 µL of incubation buffer as described above and repeat for a total number of 4 washing steps.

D. Detection

1. Remove the buffer and incubate the wells with 50 µL of anti-His-HRP antibody, diluted 1:2,000 in incubation buffer, statically for 1 h in a 24°C incubator.
2. Wash four times as described above with 200 µL of incubation buffer.
3. Add 150 µL of *o*-Phenylenediamine dihydrochloride to each well and leave the plate in a 24°C incubator without shaking for 30 min in the dark.
3. Record at 450 nm using a plate reader.

Data analysis

The experiment was replicated a minimum of three times, and each experiment had three technical replicates. Data were imported into Microsoft Excel software and baseline corrected with the incubation with PBS alone samples. Sample data for ChiA, NttE (negative control) and SsIE (positive control) is given below for binding to type II porcine stomach mucin (also see Figure 4C, Rehman *et al.*, 2020). Excel analysis provided standard deviation values and a two-tailed Student's *t*-test function was used to test for significant differences between groups tested. Only *P*-values less than 0.05 were determined to be significant.

Sample Data (Table 2)

Table 2. Example data for protein binding to type II stomach mucin extracts

	Rep1	Rep2	Rep3	Mean (PBS)	PBS subtracted Rep 1	PBS subtracted Rep 2	PBS subtracted Rep 3	Mean (mucin)	Stdev (mucin)
ChiA_PBS	0.239	0.190	0.181	0.203	-	-	-	-	-
NttE_PBS (-)	0.084	0.074	0.062	0.073	-	-	-	-	-
SsIE_PBS (+)	0.130	0.164	0.156	0.150	-	-	-	-	-
ChiA_mucin	0.933	0.846	0.900	-	0.730	0.643	0.697	0.690	0.044
NttE_mucin (-)	0.138	0.119	0.151	-	0.065	0.046	0.078	0.063	0.016
SsIE_mucin (+)	0.718	0.912	0.910	-	0.568	0.762	0.760	0.697	0.111

Recipes

1. **Incubation buffer** (prepare fresh and store at 4°C)
 - a. Mix/dissolve 0.5 g BSA and 0.5 mL Tween-20 for every 1 L of PBS buffer
 - b. Cool to 4°C
2. **50 mM Carb/Bicarb Buffer** (can be prepared in advance and stored at room temperature)
 - a. Dissolve 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ in 1 L of deionized water
 - b. Adjust the pH to 9.6 using HCl or NaOH

3. Mucin solution (can be prepared the day before and stored at 4°C)

- a. Stir for 30 min to dissolve 10 mg of either type II or type III porcine stomach mucin in 100 mL of 50 mM Carb/Bicarb buffer above.
- b. Autoclave for 15 min at 135°C to sterilize and to further dissolve the mucin*.

*Note: Mucin is difficult to dissolve and will not fully go into solution by mixing alone; thus, autoclaving is required to get a homogeneous solution.

4. Blocking buffer (can be prepared the day before and stored at 4°C)

- a. Mix/dissolve 0.1 g BSA and 0.5 mL Tween-20 for every 1 L of PBS buffer
- b. Cool to 4°C

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Competing interests

NPC and JAG have a pending patent application (63/005,592) that describes the use of ChiA for therapeutic applications.

Ethics

No human or animal subjects were used in this protocol.

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Measurements of Root Colonized Bacteria Species

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Abstract

Root-associated bacteria are able to influence plant fitness and vigor. A key step in understanding the belowground plant-bacteria interactions is to quantify root colonization by the bacteria of interest. Probably, genetic engineering with fluorescence markers is the most powerful way to monitor bacterial strains in plant. However, this could have some collateral problems and some strains can be challenging to label. In this sense, bacterial inoculation under properly controlled conditions can enable reliable and reproducible quantification of natural bacterial strains. In this protocol, we describe a detailed procedure for quantification of root-associated bacteria. This method applies non-aggressive samples processed with morphological identification and PCR-based genetic fingerprinting. This easy-to-follow protocol is suitable for studying bacterial colonization of plants grown either in artificial medium or in soil.

Keywords: Plant-bacteria interaction, Rhizobacteria, PGPR, Pathogen, Colonization, Root, Soil

This protocol was validated in: Nat Plants (2020), DOI: 10.1038/s41477-020-0707-2

Background

Plants naturally live with various soil bacteria in the rhizosphere, which refers to a thin layer of soil adhering to the roots. While some rhizobacteria have no observable effects on plants, others are either pathogens that cause detrimental effects or growth-promoting rhizobacteria (PGPR) that promote plant vigor (Mendes *et al.*, 2013; Olanrewaju *et al.*, 2019). The capacity of bacterial pathogens or PGPR to impact plant growth is tightly correlated with their level of bacterial root colonization. Therefore, the investigation of bacterial root colonization is an important stepping stone to understanding the belowground plant-bacteria interactions.

The abundance of bacteria strain can be assessed by visualization of fluorescence signals by modifying them to express a transgenic marker gene encoding the fluorescent protein such as GFP (Rochat *et al.*, 2010; Krzyzanowska *et al.*, 2012; Saad *et al.*, 2018). The abundance of bacteria strain can also be measured by PCR-based amplification of the bacterial genomic DNA (Maciá-Vicente *et al.*, 2009; Mendis *et al.*, 2018). These two methods detect the bacterial strain of interest regardless of the presence or absence of other bacterial species; nevertheless, both methods have their potential limitations. The fluorescence-assisted detection may be limited by technical difficulties during genetic transformation, by the possibility that the introduction of the transgene may interfere with wild-type bacterial behavior, and by the risks posed to environmental safety. On the other side, PCR-based bacterial detection requires that levels of the target DNA templates be above the PCR detection limits, and that the target sequence be specific enough to represent the bacterial strain of interest only.

In addition to the fluorescence-based and the PCR-based methods, root-associated bacteria can also be quantified by *in vitro* culturing. This increases the detection limits, followed by counting the colony-forming units (CFUs), if the bacterial strain of interest is culturable with an artificial growth medium. With proper experimental setups, the method of counting CFUs is efficient, affordable, reliable, and reproducible for quantification of root-associated bacteria. By using *Arabidopsis thaliana* and *B. megaterium* YC4-R4 as a model system for studying plant-bacteria interactions, herein we describe a protocol for bacteria quantification based on counting CFUs from *in vitro* bacterial cultures. This protocol, which includes an optional genetic fingerprinting step by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, has been applied to different bacterial species in studies where plants were either grown in artificial medium or in soil, and under control or stress conditions (Vilchez *et al.*, 2020). In addition to studying root-associated bacteria, this methodology can be applied to studies involving other types of plant organs or other types of bacteria-host systems under optimized conditions.

Materials and Reagents

1. 1.5 mL Eppendorf tubes (ShangYu Yite Plastic Co., Ltd, catalog number: MCTB015, or similar)
2. 15 mL tubes
3. Surgical blade and scalpel (Qingdao Sinoland International Trade Co., Ltd, catalog number: SS-002021, or similar)
4. Circular-shape (9 × 1.5 cm) and square-shape (56 × 35 × 30 cm) Petri dishes (ShangYu Yite Plastic Co., Ltd, catalog number: PD0009, or similar; Haimen Laiboreike Experiment Instrument Manufacturing Co., Ltd, catalog number: LB077, or similar)
5. Disposable tissue grinder plastic pestle for 1.5 mL tubes (Corning, Axygen®, catalog number: PES-15-B-SI, or similar)
6. Inoculation Loops (Renon Laboratory Experiment Co., Ltd, catalog number: 52150000 or similar)
7. Tape (3M Micropore™, 1530C-0, or similar)
8. Seeds of *Arabidopsis thaliana*
9. Murashige and Skoog Basal Salt Mixture (MS) (Sigma-Aldrich, catalog number: M5524)
10. Tryptone (Sigma, catalog number: T7293)
11. Yeast extract (Sigma, catalog number: Y1625)
12. NaCl (Sangon Biotech, catalog number: A501218)
13. NaOH or KOH (Sangon Biotech, catalog numbers: A100583 and A610441)
14. Agar (Sigma, catalog number: L2897)

15. Agarose (Sigma, catalog number: A9539)
16. RedSafe™ Nucleic Acid Staining Solution (Invitrogen, catalog number: S33102)
17. 2K Plus Ladder (Transgenbiotech, Trans2K® Plus DNA Marker, catalog number: BM121)
18. Double-distilled sterilized H₂O
19. 100% Ethanol (Sangon Biotech, catalog number: A500737)
20. Bleach (commercial 5% NaClO diluted 1:5 with water)
21. DNA extraction kit (Qiagen DNA Blood&Tissue, Qiagen, catalog number: 69504 or similar)
22. Master Mix solution for PCR (Transgenbiotech, 2× EasyTaq® PCR SuperMix, AS111-01)
23. Enterobacterial Repetitive Intergenic Consensus fingerprinting and 16S rRNA PCR amplification primers:
ERIC (5'-ATGTAAGCTCCTGGGGATTAC-3')
27F (5'-AGAGTTGATCMTGGCTCAG-3')
1492R (5'-TACGGYTACCTTGTACGACTT-3')

Equipment

1. Stainless steel tweezer with long fine point (Labdirect, catalog number: SA-Q2090 or similar)
2. Analytical balance (Sartorius, Practum224-1S or similar)
3. Mechanical pipettes (Eppendorf Research Plus, P1000, P100 and P10, or similar)
4. Benchtop vortex mixer (DragonLab MX-S, or similar)
5. Benchtop centrifuge (Eppendorf, Centrifuge 5810R with A-4-62 Model Rotor, or similar; up to 4,000 rpm/3,220 × g)
6. Clean bench (BCM-1000A Biological Clean Bench, Airtech, or similar)
7. Shaker incubator (Eppendorf, New Brunswick™ I26 Stackable Incubator Shakers, or similar)
8. Rolling incubator (Kylin-Bell Lab Instruments Co., Ltd., Qilinbeier QB-128, or similar)
9. Autoclave
10. Pasteur oven
11. Plant growth chamber (Percival CU36L5 or similar) or growth room.
12. Culture oven (Shanghai JingHong DHG-9038A, or similar)
13. Electrophoresis devices (Bio-Rad, PowerPac™ Basic Power Supply + Wide Mini-Sub Cell GT Cell, or similar)
14. A gel imaging system (Bio-Rad, ChemiDoc XRS+™ Gel Imaging System, or similar)
15. Thermocycler (Bio-Rad, T100 Thermal Cycler, or similar)

Procedure

A. Bacterial culture using *B. megaterium* YC4-R4 as an example

1. Refresh YC4-R4 from glycerol stock on LB-Agar plate and incubate at 37°C for 24 h*/**.
2. Take a single colony to inoculate 5 mL LB medium (*in vitro*) (recommended 15 mL tubes). Incubate overnight in a shaker (220 rpm, 37°C). For soil inoculation, scale up by using 1 mL of pre-culture per liter of fresh culture (final volume depends on soil initial moisture and pot volume)*.
3. Prepare 0.45% NaCl solution by mixing 4.5 g per liter of ddH₂O. Autoclave for 20 min at 121°C. Cool to room temperature before use.
4. Centrifuge the culture at 3,220 × g for 10 min. Resuspend the pellet in the same volume of 0.45% NaCl. OD₆₀₀~0.8-1.1 (log growth phase; generally, equals to 10⁶-10⁸ CFU/mL) is recommended.

Notes:

a. *This step shall be done under sterile conditions.

b. **For tests using antibiotic-resistant strains, LB plates may be prepared with the appropriate antibiotics.

B. Prepare bacteria growth medium

1. Mix 10 g of tryptone, 5 g of yeast extract, and 10 g NaCl per liter of ddH₂O.
2. For making LB agar plates, add 15 g agar per liter.
3. Autoclave for 20 min at 121°C. For LB agar plates, pour the liquid medium into Petri dishes and allow it to cool down and solidify.

C. Prepare plant growth medium

1. Mix MS powder in half strength (2.21 g) and agar (0.7% and 1% for circular and square plates, respectively, depending on test requirements) per liter of ddH₂O.
2. Adjust pH to 5.7 with NaOH or KOH.
3. Autoclave for 20 min at 121°C. Pour the medium into the Petri dishes and allow it to cool down and solidify.

D. Seed sterilization and plant growth conditions

1. Mix seeds with absolute ethanol in 1.5 mL tubes and keep in agitation for 1 min. For 100 seeds, 1 mL of ethanol is recommended.
2. Discard ethanol and add 20% bleach solution (from commercial bleach stock). For 100 seeds, 1 mL of 20% bleach solution is recommended. Agitate in a spinner wheel for 10-15 min.
3. Discard bleach and wash three times with sterile ddH₂O in a clean bench*.
4. Plant the seeds on MS medium one by one with the aid of pipet tips*.
5. Stratify the seeds at 4°C for 48 h. After stratification, place the plates vertically in the growth chamber for 14 days under the following conditions: 22°C; 12 h light/12 h dark cycle; 40% relative humidity; and up to 155 $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ light intensity.

Note: *This step shall be done under sterile conditions.

E. In vitro colonization test

1. Under sterile conditions, place 15-20 14-days-after-germination (DAG) seedlings grown in $\frac{1}{2}$ MS 1% agar (in squared Petri plates) per replica in a 1.5 mL sterile tube (volume of sterile tube depends on plant size). Add 1 mL of bacteria in 0.45% NaCl solution.
2. Incubate the tube overnight on a shaker (220 rpm, 26°C*).
3. Discard the bacterial solution. Place the seedlings on a sterile surface, such as sterilized aluminum foil or paper, or a Petri dish. Cut roots with sterile razor blades and keep them in 1.5 mL tubes.
4. Sterilize the root surface by adding 1 mL of 75% ethanol, treating for 7-10 min with continuous agitation (spin wheel or equivalent).
5. Discard the ethanol and wash the roots for three times with sterile ddH₂O.
6. Discard the ddH₂O and grind roots with sterile pistils (for 1.5 mL tubes).
7. Add 1 mL of 0.45% NaCl to the tube, homogenize by vortexing, and prepare serial dilutions (usually 5 times 10-fold dilution should be enough).
8. Plate drop by drop (10 drops of 10 μL approx. up to a total volume of 100 μL) each dilution in LB-agar plates, let them dry for 2 min and incubate at 30°C** for 24 h (Figure 1).

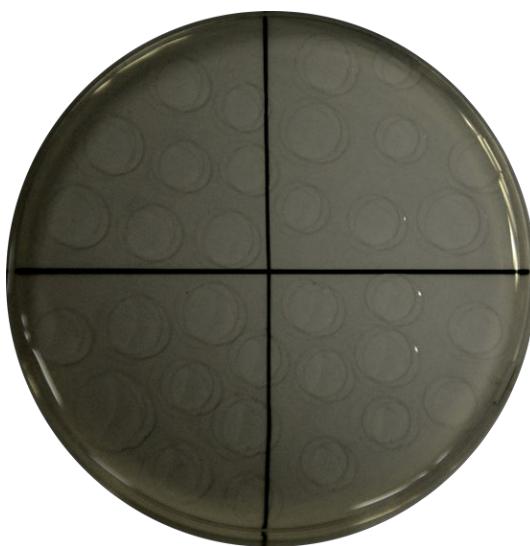


Figure 1. Example of drop-by-drop seeding of a serial dilution in a quarter-divided growth plate.

Drops are carefully placed on the dry growth medium plate to avoid interferences.

9. Keep and centrifuge the original solution at the maximal speed. Once root debris is pelleted, discard the supernatant and fully dry at 60°C in an oven for 48 h and record this weight as the dry weight (DW), by using Pasteur oven.
10. Count bacteria colony numbers and calculate the colony forming units (CFUs) by taking into account the dilution and plated volume. Normalize the CFU values with root DW to obtain the colonization rates.
Colonization rate = (CFUs × dilution factor)/mg of root DW.

Notes:

- a. All work shall be done under sterile conditions.
- b. *This temperature has been tested to ensure non-stressing conditions for plants; at the same time, bacteria growth under the same temperature should also be considered to avoid imposing stress to the bacteria.
- c. **The temperature used herein is optimal for colony-counting of the example bacteria. It is highly recommended to optimize the temperature for different strains in order to avoid overgrowth/overlapped colonies.

F. Determination of colonization ratio in soil-grown plants

1. Grow *Arabidopsis* seedlings as described above.
2. Prepare soil based on the conditions required. In our tests, soil is prepared by meshing a mix 1:3 (v:v) of vermiculite and soil substrate. To minimize unwanted microbes in the soil, the soil needs to be tyndalized (75°C for 3 h + cooling interval for 10 h + 75°C for another 3 h). Transfer 5 DAG seedlings to the soil.
3. Inoculate with bacteria in 0.45% NaCl solution. As reference, in a regular 0.4 L pot, inoculate 50 mL of bacteria solution around planted seedlings.
4. Allow the plants to grow under conditions as required by the desired tests (e.g., with or without certain abiotic stress).
5. Harvest the roots (usually 20-40 mg of roots) at the time when colonization quantification is desired. For root harvesting, manually detach the soil around each root (with the help of tweezers) and then use a soft tissue to detach the smallest and most adhered pieces of soil. Fine soil particles must be removed by 3 × ddH₂O washing*. Once roots are clean, follow indications for sterilizing, grinding, preparing dilutions, and counting CFUs in Part E.

*Note: *This step shall be done under sterile conditions.*

G. Strain isolation and identification

1. Although the soil was tyndalized, typically Part F will yield a small portion of bacteria other than the inoculated strain. To confirm the identity of the inoculated strain and to calculate its proportion in the total bacterial population, first classify bacterial colonies by morphology (use <https://microbeonline.com/c colony-morphology-bacteria-describe-bacterial-colonies/> as reference), then use sterile toothpicks to pick single colonies with similar morphology and transfer them to liquid LB medium for culturing. Usually, 20-30 colonies should be randomly sampled to avoid bias (Figure 2).



Figure 2. An example of colonies with different morphologies in a regular root colonization quantification process.

Different morphologies were selected by color, size, or brightness of the colonies. The colour of the arrows indicates a pre-group of strains: according to the following criteria: red – intense yellow color; blue – white color, big size; orange – white color, small size; and green – beige color, matte surface.

2. Culture the individual colonies in 5 mL of liquid LB on a shaker (220 rpm, 30°C) for 24 h.
3. Centrifuge the culture (suggestion in bench centrifuge: 3,220 × g, 10 min), discard supernatant, and extract DNA from pellets by using a high-performance method. Here, a DNA extraction kit is suggested (see methods).
4. Amplify and sequence 16S rRNA genes with the 27F (5'-AGAGTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3') (or equivalent), and BLAST the sequence to identify isolated species.
5. To further distinguish the inoculated strain from other strains of the same species, perform PCR with ERIC primer (5'-ATGTAAGCTCCTGGGGATTCA-3') to generate fingerprinting phenotype patterns for each strain. Use the stock strains employed in the test as the reference. Run the samples in a 2% agarose gel to maximize band separation. Use GelJ to compare the patterns with the reference and obtain similarity indexes. Figure 3 includes a representative gel image output.

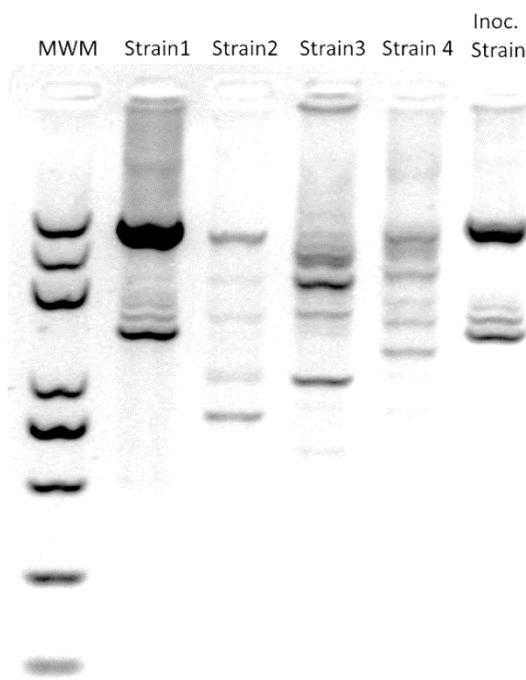


Figure 3. Example of a representative ERIC-PCR gel image output (modified from Vilchez *et al.*, 2020). MWM: molecular weight marker; Strains 1 to 4 were recovered from roots in inoculated soil; Inoc. Strain: originally soil-inoculated strain.

Data analysis

Each test should be carried out with at least three replicates per condition prepared as indicated above (*in vitro* or in soil). Results will be considered statistically different when P -value < 0.05 according to Student's *t*-test. The relative colonization rates are calculated as: $RC = (\text{colony numbers} \times \text{dilution factor})/\text{mg of root DW}$.

Notes

1. If applying this method to other plant species, scale the recipe to fit the plant size. For grinding samples, it is recommended to use 1.5 mL tubes and the matching pistils. Automatic grinding may be performed at low vibration frequencies. In the case of using mechanical disruption devices such as TissueLyser, use low frequencies of disruption in order to avoid overheating and mechanical stress that may cause disruption of bacterial membranes. Mechanical disruption at high frequencies is used to extract total DNA from soil, sludge, or other complex samples. Aggressive grinding/disruption methods increase the likelihood of introducing biases in the final counting and identification of bacterial strains.
2. The ethanol-based sterilization process has been tested in roots of Arabidopsis and tomato as the reference plants. In the case of Arabidopsis (Figure 4), 7-10 min (about 5 min for longer and thicker roots, as in tomato) was assessed as optimal root surface sterilization time (these tests were performed with Gram+ bacteria; consider shorter time for Gram-). Longer incubation periods could interfere with the quantification of the colonizing bacteria.

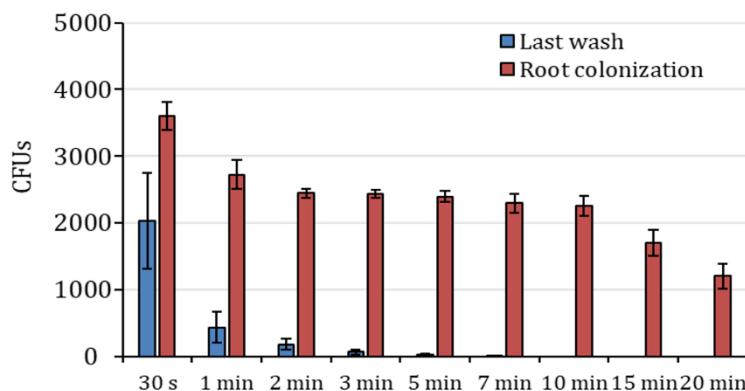


Figure 4. Colony counts after ethanol treatment in *Arabidopsis* roots.

Colony forming units (CFUs) counted in the last wash solution after treatment with ethanol (blue columns) and respective root colonization counting (red columns). The X-axis indicates ethanol treatment time. n=3, data are represented as mean \pm SE.

3. Nevertheless, it should be noted that this set up is for *Arabidopsis* plants. Sterilization time depends on the type of plant and bacterial population tested, so it is highly recommended to optimize sterilization time depending on the actual experimental set up.

Acknowledgments

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Competing interests

The authors declare no competing interests.

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A Sensitive and Specific PCR-based Assay to Quantify Hepatitis B Virus Covalently Closed Circular (ccc) DNA while Preserving Cellular DNA

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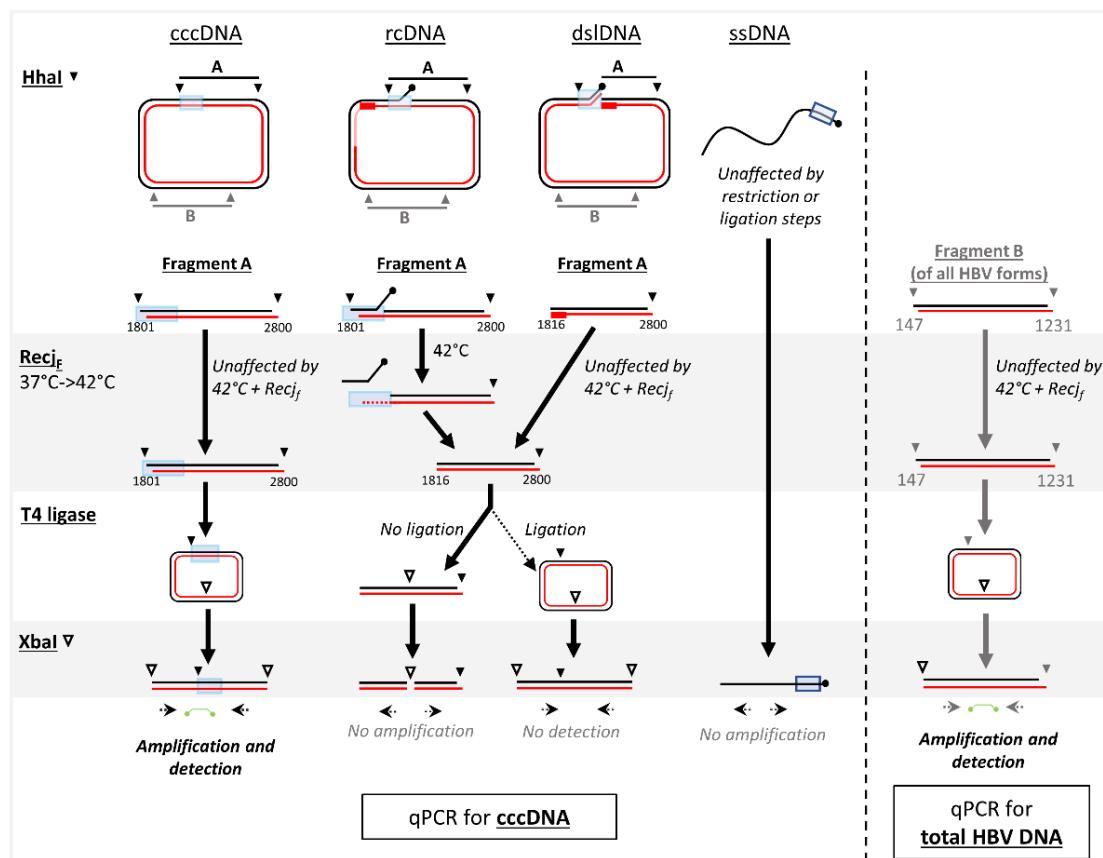
Abstract

Hepatitis B virus (HBV) is the major cause of liver diseases and liver cancer worldwide. After infecting hepatocytes, the virus establishes a stable episome (covalently closed circular DNA, or cccDNA) that serves as the template for all viral transcripts. Specific and accurate quantification of cccDNA is difficult because infected cells contain abundant replicative intermediates of HBV DNA that share overlapping sequences but arranged in slightly different forms. HBV cccDNA can be detected by Southern blot or qPCR methods which involve enzymatic digestion. These assays are laborious, have limited sensitivity, or require degradation of cellular DNA (which precludes simple normalization). The method described in this protocol, cccDNA inversion quantitative (cinq)PCR, instead uses a series of restriction enzyme-mediated hydrolysis and ligation reactions that convert cccDNA into an inverted linear amplicon, which is not amplified or detected from other forms of HBV DNA. Importantly, cellular DNA remains quantifiable during sample preparation, allowing normalization and markedly improving precision. Further, a second linear fragment (derived from enzymatic digestion of a separate region of the HBV DNA genome and is present in all forms of HBV DNA) can be used to simultaneously quantify total HBV levels.

Keywords: Hepatitis B virus, cccDNA, Hepcludex®, Bulevirtide, Myrcludex B, cinqPCR, DNA nick, DNA repair, Viral persistence

This protocol was validated in: Antiviral Res (2020), DOI: 10.1016/j.antiviral.2020.104865

Graphical Abstract:



Selective detection of HBV cccDNA and total HBV DNA using cinqPCR (Reproduced from Tu *et al.*, 2020a).

Background

Hepatitis B virus (HBV) is a small enveloped virus, which encapsidates a partially double-stranded circular DNA genome, the so-called relaxed circular (rc) DNA. Upon infection of human hepatocytes, the nucleocapsid is transported to the nucleus wherein the rcDNA genome is converted to covalently closed circular (ccc) DNA. This episomal form is highly stable and maintains chronic HBV infection (Tu *et al.*, 2020b). It serves as template for all viral transcripts and the pre-genomic RNA. Elimination of the cccDNA would lead to a complete cure of a chronic hepatitis B infection. Thus, the reliable quantification of cccDNA is key to developing strategies for HBV cure.

In any given infected hepatocyte, HBV DNA exists in different forms (rcDNA, double stranded linear (ds) DNA, single stranded (ss) DNA, and cccDNA) that share overlapping sequences, but have different structures (Nassal, 2015). Thus, differentiation of cccDNA (which is present in low numbers) from other HBV DNA forms is technically challenging. Southern blot is the gold standard method used to differentiate cccDNA from other forms, though it has low throughput and poor sensitivity. PCR-based methods are more sensitive and can be applied in a medium-throughput manner. Current protocols include an exonuclease treatment (with T5 exonuclease, plasmid-safe DNase, or a combination of ExoI and ExoIII) to digest all DNA species that are not covalently closed (including cellular DNA) (Luo *et al.*, 2017; Allweiss *et al.*, 2018; Qu *et al.*, 2018). By contrast, the method described in this protocol takes advantage of particular restriction sites within the HBV DNA sequence (see graphical abstract) to selectively convert cccDNA into an amplifiable form. Therefore, our method allows the normalization to a single-copy cellular gene and total HBV DNA can be detected from the same sample, dramatically improving precision,

sensitivity, and accuracy.

Materials and Reagents

Note: All stored at room temperature unless otherwise stated.

1. 24-well cell culture plates (Corning, catalog number: 3527)
2. 1.5 mL Eppendorf tubes (Sarstedt, catalog number: 72706)
3. Sealing Mats for 96-Well PCR Plates, reusable (Bio-Rad, catalog number: 2239442)
4. TwinTec PCR plates, 96 semi-skirted (Eppendorf, catalog number: 0030128.575)
5. Aluminium Foil Seals for PCR and QX100 ddPCR applications (Bio-Rad, catalog number: 181-4040)
6. P20 racked barrier LTS tips (Rainin, catalog number: RT-L20F)
7. P200 racked barrier LTS tips (Rainin, catalog number: RT-L200F)
8. HBV-susceptible cells, *e.g.*, HepG2-NTCP cells (Ni *et al.*, 2014) (maintain in 37 °C incubator at 5% CO₂ supply and 95% humidity)
9. HBV genotype D virus stock (store at -80°C)
10. Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich, catalog number: D8537)
11. Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, catalog number: 41965039, store at 4°C)
12. Fetal bovine serum (FBS, Sigma-Aldrich, catalog number: S0615, store at -20°C)
13. L-Glutamine (Life Technologies, catalog number: 25030024, store at -20°C)
14. Penicillin (10,000 U/mL) Streptomycin (10 mg/mL) (Life Technologies, catalog number: 15140-122, store at -20°C)
15. Dimethylsulfoxide (DMSO, Merck, catalog number: 102950)
16. Polyethyleneglycol (PEG 8000, 40% solution in PBS, Sigma-Aldrich, catalog number: 89510)
17. Trypsin-EDTA solution (Sigma-Aldrich, catalog number: T3924, store at 4°C)
18. NucleoSpin® Tissue kit (Macherey-Nagel, catalog number: 740952)
19. Multichannel pipette (Eppendorf, catalog number: 3125000036)
20. 10× Cutsmart buffer (NEB, catalog number: B7204, store at -20°C)
21. *Hha*I (NEB, catalog number: R0139, store at -20°C)
22. PCR-grade H₂O (*e.g.*, B. Braun Melsungen, for injection purposes)
23. RecJ_f (NEB, catalog number: M0264, store at -20°C)
24. T4 DNA Ligase (NEB, catalog number: M0202, store at -20°C)
25. 10 mM Molecular-grade ATP (NEB, catalog number: P0756, store at -20°C)
26. *Xba*I (NEB, catalog number: R0145, store at -20°C)
27. DNAZap PCR DNA Degradation Solutions (Thermo Scientific, catalog number: AM9890, store at 4°C)
28. VIC-labelled TaqMan™ Copy Number Reference Assay for the human RNase P gene (Applied Biosystems, catalog number: 4403328, store at -20°C)
29. ddPCR Supermix for Probes (Bio-Rad, catalog number: 1863010, store at -20°C)
30. Primers/probe for cccDNA detection (ordered from Eurofins genomics, store at -20°C)
 - a. cccDNA for: 5'-CACTCTATGGAAGGCAGGT-3'
 - b. cccDNA rev: 5'-ATAAGGGTCGATGTCCATGC-3'
 - c. cccDNA probe: 5'-FAM- AACACATAGCGCACCAAGCA-BHQ1-3'
31. Primers/probe for total HBV DNA (ordered from Eurofins genomics, store at -20°C)
 - a. Total HBV for: 5'-GTGTCTGCGCGTTTATCA-3'
 - b. Total HBV rev: 5'- GACAAACGGGCAACATACCTT-3'
 - c. Total HBV probe: 5'-FAM-TGAGGCATAGCAGCAGGATG-BHQ1-3'
32. DG8 cartridges (Bio-Rad, catalog number: 186-4008)
33. DG8 gaskets (Bio-Rad, catalog number: 186-3009)
34. Droplet Reader Oil (Bio-Rad, catalog number: 186-3004)
35. Droplet generation oil for probes (Bio-Rad, catalog number: 186-3005)
36. Growth medium (*see Recipes*)

37. Infection medium (see Recipes)
38. Ligation mix (see Recipes)
39. Linearization mix (see Recipes)

Equipment

1. Pipette for Rainin P20 tips (Rainin, catalog number: 17014392)
2. Multichannel pipette for Rainin P200 tips (Rainin, catalog number: 17013805)
3. Thermo cycler (Analytik Jena Biometra, FlexCycler²)
4. QX200TM Droplet generator (Bio-Rad, catalog number: 1864002)
5. PX1 PCR plate sealer (Bio-Rad, catalog number: 1814000)
6. C1000 TouchTM Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad, catalog number: 1851197)
7. QX200TM Droplet Reader (Bio-Rad, catalog number: 1864003)

Software

1. QuantaSoft (Bio-Rad)
2. Microsoft Excel
3. Prism (GraphPad)

Procedure

A. Sample preparation

1. Seed 2.5×10^5 cells per well in a 24-well format in growth medium one day before infection.
2. Infect cells overnight with HBV virus stock in infection medium containing 4% PEG 8000 (Ni *et al.*, 2014).
3. Wash cells twice with PBS at room temperature (RT) and maintain in infection medium until desired harvest time.
4. Wash cells with 500 μL PBS at RT and add 100 μL trypsin.
5. Incubate at 37°C until the cells detach (~5 min).
6. Resuspend the cells with 900 μL cold medium and transfer to a 1.5 mL Eppendorf tube.
7. Centrifuge for 5 min at 500 $\times g$ at RT and remove the supernatant.

Optional: Freeze the pellet at -20°C until further use.

8. Extract total DNA from the cell pellets using NucleoSpin[®] Tissue kit according to the manufacturer's instructions, but elute in 50 μL elution buffer pre-warmed to 70°C.

B. Inversion reaction

1. Clean a silicon sealing mat using the DNA Zap solutions, rinse in deionised water, and let dry on a paper towel.
2. Prepare the restriction digestion reaction in a 96-well plate containing 10 μL of extracted total DNA, 2 μL of 10 \times Cutsmart buffer, 0.5 μL *Hha*I, 0.25 μL RecJ_f, and 7.25 μL H₂O.
3. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
4. Seal with the clean and dry silicon mat.

5. Run the following program in a thermo cycler: 3 cycles of 15 min at 37°C and 15 min at 42°C, 20 min at 80°C, and hold at 16°C.
6. Remove the mat carefully and add 10 µL of the ligation mix.
7. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
8. Incubate in a thermo cycler at 16°C for 2 h, 80°C for 20 min, and hold at 16°C.
9. Remove the mat carefully and add 5 µL of the linearization mix.
10. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
11. Incubate in a thermo cycler at 37°C for 60 min, 80°C for 20 min, and hold at 16°C.

Optional: Store samples at -20°C.

C. Droplet generation and reading

1. Dilute the sample 2.5-fold by adding 52.5 µL H₂O.
2. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
3. Prepare two reactions for each sample: (1) cccDNA and RNaseP cellular control and (2) total HBV DNA and RNaseP cellular control. Prepare ddPCR mix in a 96-well plate (22 µL per reaction) (see Table 1).

Table 1. ddPCR mix composition for each reaction

Reagent	Concentration	µL
ddPCR Supermix for probes	2×	11
TaqMan™ Copy Number Reference Assay (VIC-labelled RNaseP)	10×	1.1
Forward primer (cccDNA or total HBV DNA)	100 µM	0.033
Reverse primer (cccDNA or total HBV DNA)	100 µM	0.033
FAM-labelled probe (cccDNA or total HBV DNA)	100 µM	0.033
H ₂ O		4.5
Diluted template		5.5

4. Use microplastic-free Rainin tips. Generate droplets on the ddPCR droplet generator:
 - a. Add 20 µL of the ddPCR mix to the sample chambers of a cartridge.
 - b. Add 70 µL of droplet generation oil using a multichannel pipette in the oil chamber.
 - c. Attach the rubber gasket, place the cartridge in the droplet generator, and run the droplet generation.
 - d. Gently transfer 42 µL of the generated droplets using a multichannel pipette to a TwinTec 96-well plate.
 - e. Repeat Steps C4a to C4d until all samples are loaded into the TwinTec 96-well plate.
 - f. Seal the plate with an aluminum foil seal using the plate sealer.
5. **Directly after sealing**, run PCR in deep-well PCR machine with the following program: 10 min at 95°C; 40 cycles of 10 s at 95°C, 15 s at 54°C, and 20 s at 68°C; 10 min at 95°C, and hold at 12°C.
6. Transfer the TwinTec 96-well plate to the QX200 Droplet Reader.
7. Set up template using the Quantasoft software (as per manufacturer's user guide), reading droplets using channels for FAM (Channel 1) and VIC (Channel 2).
8. Define thresholds for positive and negative droplets based on positive and negative controls, after which the software will automatically quantify values for cccDNA per RNaseP and total HBV DNA per RNaseP.

Data analysis

In the Quantasoft software, we use the 2D graph to draw thresholds for HBV DNA and for the RNaseP positive droplets (Figure 1). The thresholds are gated separately for all the total HBV DNA and all the cccDNA assays. For the data presentation we use the ratios total HBV DNA/RNaseP and cccDNA/RNaseP of each sample with the PoissonRatioMin and PoissonRatioMax values giving a 95% confidence interval of the technical error of the ddPCR.

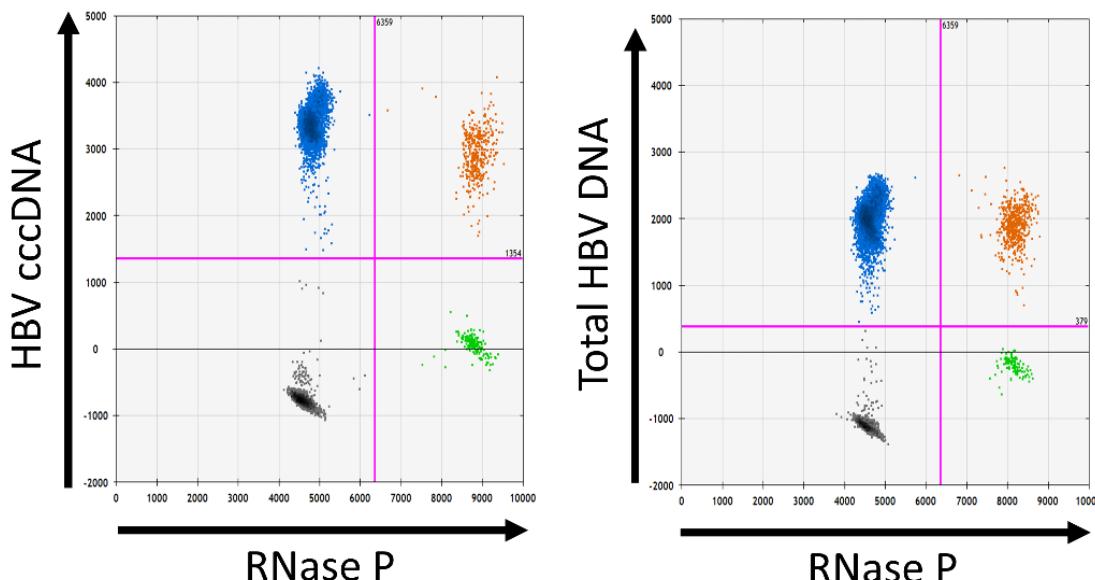


Figure 1. Data analysis using QuantaSoft.

The fluorescence intensity was used to separate droplets containing amplicons detecting: HBV cccDNA (left) or total HBV DNA (right), based on FAM fluorescence (y-axis); and RNaseP, based on VIC fluorescence (x-axis). The thresholds for positive signals (pink) were gated based on positive and negative control reactions (Reproduced from Tu *et al.*, 2020a).

Notes

1. We have used this protocol with DNA extracted from cells infected in plate formats from 12-well to 96-well. In 12-well plates, we advise to elute the DNA in twice the volume.
2. For the inversion, we recommend an input of <2 µg of total DNA extract. We also recommend adding 200 ng of carrier DNA (*e.g.*, unrelated plasmid DNA) if the total DNA input is <200 ng.
3. We usually perform HBV infections with a multiplicity of viral genome equivalents of 200. However, infection can also be performed with a smaller inoculum as the assay is sensitive enough to detect very low numbers of infected cells (Tu *et al.*, 2020a).
4. This protocol can also be used with Taqman-based qPCR instead of ddPCR.
5. This assay is only compatible with HBV sequences from genotype D which is used in most *in vitro* HBV infection assays. Unfortunately, it does not work with other genotypes due to variation in *Hha*I restriction sites. Most *in vitro* assays with HBV are performed with the genotype D strain, this shortcoming does not allow the analysis of clinical samples.
6. Recommended controls for optimization and quality control:
 - a. **Positive control to show detection efficiency** (total HBV DNA quantification should give similar number as cccDNA quantification): HBV circDNA (Mutz *et al.*, 2018) or HBV 1.1 overlength plasmid spiked into DNA extracted from uninfected cells.

- b. **Negative control to show selective quantification of cccDNA:** DNA extracted from heparin-purified virus (Seitz *et al.*, 2016) spiked into DNA extracted from uninfected cells.
- c. **Negative control to determine signal from virus inoculum during cell infection:** Treatment of cells with an entry inhibitor (*e.g.*, Myrcludex B) prior to HBV infection (Donkers *et al.*, 2017).
- d. **No template control and uninfected cell control to determine level of non-specific amplification.**

Recipes

1. Growth medium

500 mL of DMEM
50 mL of FBS
5 mL of L-Glutamine
5 mL of Penicillin (10,000 U/mL) and Streptomycin (10 mg/mL)

2. Infection medium

49 mL of Growth medium
1 mL of DMSO

3. Ligation mix (10 µL per sample)

3 µL of 10 mM ATP
1 µL of 10× NEB Cutsmart buffer
0.5 µL of T4 ligase
5 µL H₂O

4. Linearization mix (5 µL per sample)

0.5 µL of 10× Cutsmart buffer
0.5 µL of *Xba*I
4 µL H₂O

Acknowledgments

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Competing interests

Stephan Urban is co-applicant and co-inventor on patents protecting HBV preS-derived lipopeptides (bulevirtide / Hepcludex®, formerly Myrcludex B) for their use as HBV/HDV entry inhibitors. The other authors in this study declare no competing interests.

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Screening for Lysogen Activity in Therapeutically Relevant Bacteriophages

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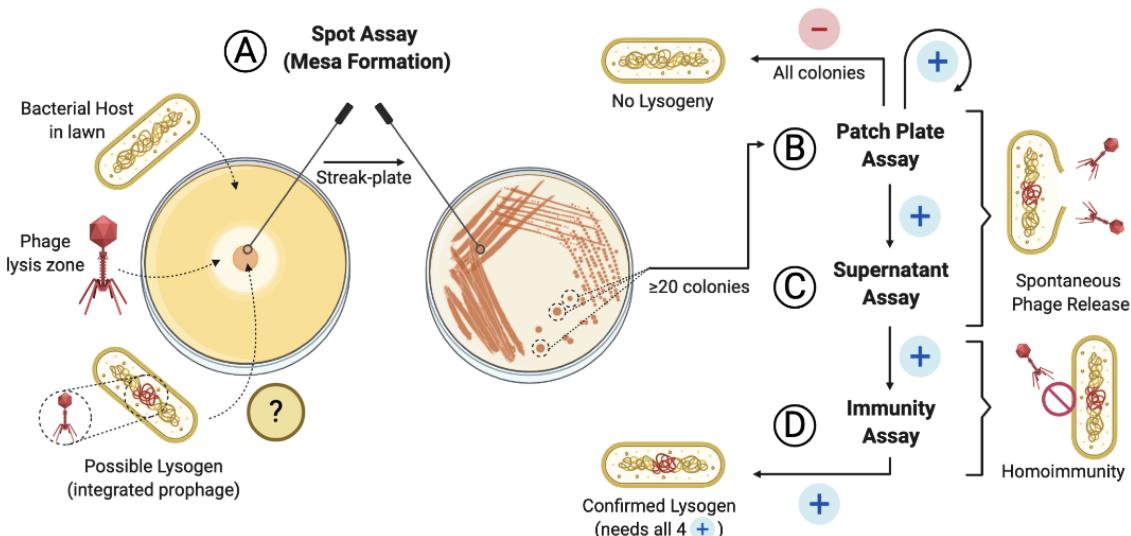
Abstract

Lysogenic phages can integrate into their bacterial host's genome, potentially transferring any genetic information they possess including virulence or resistance genes, and are therefore routinely excluded from therapeutic applications. Lysogenic behavior is typically seen in phages that create turbid plaques or possess subpar bactericidal activity; yet, these are not definitive indicators. As a result, the presence of integrase genes is often used as a hallmark for lysogenic behavior; however, the accuracy of genetic screening for lysogeny depends on the quality of the extraction, sequencing and assembly of the phage genome, and database comparison. The present protocol describes a simple phenotypic test that can be used to screen therapeutically relevant phages for lysogenic behavior. This test relies on the identification of spontaneous phage release from their lysogenized host and can be reliably used in cases where no sequencing data are available. The protocol does not require specialized equipment, is not work-intensive, and is broadly applicable to any phage with an easily culturable bacterial host, making it particularly amenable to settings with limited resources.

Keywords: Bacteriophage, Phage therapy, Lysogeny, Prophage integration, Prophages, *Acinetobacter baumannii*, *Enterococcus faecium*

This protocol was validated in: Nat Microbiol (2021), DOI: 10.1038/s41564-020-00830-7

Graphical Abstract:



Screening pipeline for lysogen activity of a given phage.

Background

The field of phage therapy, which is the clinical use of viruses to kill bacteria, has grown exponentially over recent decades (Gordillo Altamirano and Barr, 2019). Prior to their selection for therapeutic use, phages are typically subjected to a thorough characterization pipeline (Terwilliger *et al.*, 2020). Lysogenic or temperate phages are those with the capacity to integrate themselves into their host's genome, and are routinely excluded from therapeutic use due to their subpar bactericidal ability, the emergence of homoimmunity, and the potentially harmful consequences of lysogenic conversion (Fortier and Sekulovic, 2013; Hyman, 2019).

Temperate behavior is first suspected in phages that produce turbid plaques on bacterial lawns. However, plaque morphology can vary greatly depending on a variety of factors, including the growth medium used and the physiological state of the bacterial host (Ramesh *et al.*, 2019). Delayed or substandard bactericidal activity in bacterial growth assays can also serve as an indicator, but their interpretation is not always clear-cut. Alternatively, genotypic screening for genes that are essential for lysogeny, such as integrase and excisionase enzymes, are further indicators of temperate behavior (Hyman, 2019). However, extraction and sequencing of phage genomes can be challenging, and the accuracy of homology-based analyses is highly dependent on the quality of the sequencing data, assemblies, and available databases (Russell, 2018). Importantly, the presence of a predicted integrase in the genome of a phage may not be definite confirmation of the ability to lysogenize a host *in vitro* or *in vivo* (Howard-Varona *et al.*, 2017).

Here, we outline a simple protocol, modified from Pope *et al.* (2013), that evaluates the ability of a given phage to lysogenize its host *in vitro*. The assay can be reliably used in cases where no sequencing data are available. Furthermore, this protocol has the advantage of providing *confirmation* of lysogenic behavior for a given phage, instead of *prediction*, given by genome sequencing-based tests. It does not require specialized equipment and can complement analysis of plaque morphology and growth. Although we describe the protocol using an *Acinetobacter baumannii*-specific phage (Gordillo Altamirano *et al.*, 2021), we also provide data from an *Enterococcus faecium*-specific phage (Figure 1), demonstrating that the methodology is broadly applicable to any phage with a bacterial host culturable on solid media. As such, incubation and media conditions may need to be adapted to suit the host. Finally, in addition to excluding lysogenic phages from therapeutic use, the protocol can be used to produce lysogens (bacteria harboring a prophage) for downstream applications in phage biology and biotechnology research.

Materials and Reagents

1. 15-mL polystyrene tubes (In Vitro Technologies, Falcon, catalog number: FAL352095)
2. Pipettes, serological pipettes, and pipette tips
3. 10-mL sterile rimLess glass test tubes (Thermo-Fisher, Yoully, catalog number: LBSDCT13100)
4. Wire loop
5. 10-mL syringes with detachable needle (McFarlane, Terumo, catalog number: 19046TE)
6. 0.2- μ m filters, attachable to syringes (Pall, catalog number: 4612)
7. Phage to be tested, high titer lysate ($\geq 10^6$ plaque-forming units [PFU]/mL)

Note: For a protocol on phage isolation and purification, see Bonilla et al., 2016.

8. 1× phosphate-buffered saline (PBS) solution (Merck Australia, OmniPur, catalog number: US16506)
9. BactoTM Tryptone (Gibco, catalog number: 211705)
10. Granulated yeast extract (Merck Australia, Millipore, catalog number: 1037530500)
11. NaCl (Merck Australia, Supelco, catalog number: 1064040500)
12. Agar powder (Merck Australia, Millipore, catalog number: 05040)
13. Overnight culture in LB of the phage's bacterial host (see Recipes for LB medium)
14. LB medium top agar (see Recipes)
15. LB medium agar plates (see Recipes)

Note: The growth media in 13-15 can be replaced to cater for the bacterial host's growth requirements.

Equipment

1. Microwave (Panasonic, model: 32 L stainless steel, catalog number: NN-ST67JSQPQ)
2. 37°C incubator
3. Shaking platform (Thermo Scientific, model: CO₂-resistant shaker, catalog number: 88881102)
4. Bunsen burner
5. Benchtop centrifuge (Eppendorf South Pacific, model: 5810 R, catalog number: 5811000487)
6. Autoclave (Tuttnauer, model: vertical 110L, catalog number: 5050ELV-D)

Procedure

A. Coincubation of phages and bacterial hosts (spot assay)

Note: The estimated time to complete this section is 45 min of benchwork followed by overnight incubation.

1. In a sterile glass test tube, mix 1 mL bacterial culture with 3 mL molten top agar.

Note: The top agar can be liquified using a microwave. Be careful: too hot a temperature of the top agar will kill the bacterial population, resulting in a poor lawn. The top agar should be liquid but pleasantly warm to the touch (approximately 50°C).

2. Pour the mixture onto a room-temperature LB agar plate and allow to solidify.
3. Prepare two or three serial dilutions (~ 1 mL) of the high-titer phage lysate in PBS.
Note: We recommend using at least two concentrations of phage, for example 10⁸ and 10⁶ PFU/mL.
4. Spot 10- μ L drops of each phage lysate dilution onto the surface of the prepared plate.

Note: Spots of further dilutions or replicates of the same dilutions can be added to the same plate.

5. Leave the plate, lid partially on, close to a Bunsen burner until the spots are completely dry; this can take up to 30 min. Alternatively, the plates can be left to dry in a biosafety hood.
6. Incubate at 37°C for up to 4 days, with daily checks to assess the formation of “mesas” (Figure 1A) (Pope *et al.*, 2013).

Note: Mesas are zones of confluent bacterial growth in the center of the lysis spots. Bacteria growing on mesas have become resistant to the phage, possibly (but not exclusively) due to homoimmunity. While checking for mesas, also look for agar dehydration due to prolonged incubation, which could hamper bacterial viability. Desiccation can be further avoided by incubating the plates in a sealed container with a wet paper towel, creating a humid chamber.

7. Using a sterile wire loop, scrape one loopful of bacterial growth from a mesa and use it as the primary inoculum for a streak plate on fresh LB agar (see Graphical abstract). Repeat for each mesa.
8. Incubate at 37°C overnight. Assess the growth of clearly isolated colonies.

Note: Any of these colonies could contain the integrated prophage. We routinely test 10 colonies obtained from each of at least two mesas, for a total of at least 20 colonies.

B. Patch plate screen

Note: The estimated time to complete this section is 30 min of benchwork and multiple overnight incubations.

1. Label and divide two LB agar plates into a grid with as many sections as colonies to be screened. Number the sections on both plates identically from 1 to 20.

Note: A single plate can comfortably fit 20 colonies.

2. Prepare one of the plates with a lawn of bacterial host following Steps A1 and A2. The other plate is used without further preparation.
3. Using a sterile wire loop, a disposable pipette tip, or a sterile toothpick, take a random colony from the streak plate (prepared in Step A7), gently patch it onto section 1 of the LB plate, and then onto its corresponding section of the LB plate with the bacterial host lawn (Step B2).

Note: Avoid tearing the delicate agar while performing the patches.

4. Repeat Step B3 with as many colonies as needed.
5. Incubate the plates at 37°C for up to 2 days, with daily checks for interpretation.
6. To interpret the test, look closely at the plate with the host lawn. Positive colonies, those potentially harboring the prophage, will present signs of lysis (plaques, halos, loss of turbidity) on the lawn around them, which is caused by the spontaneous release of the phage (Figure 1B).

Note: If all the screened colonies are negative for the patch test, it can be concluded that the phage is unable to lysogenize the host (see Table 1). False positive results here are possible due to carry-over of phage particles from the mesa onto the streak plate (Step A7).

7. For each positive colony, return to the LB plate without the host lawn (prepared in Steps B3-B4), pick a section of the patch with a wire loop, and streak onto a fresh LB plate.
8. Incubate overnight at 37°C.
9. Pick a single colony with a wire loop and re-streak onto a fresh LB plate.
10. Incubate overnight at 37°C.

Note: You have now performed a double single-colony purification. Using colonies from this plate (B9), repeat Procedure B (Figure 1C) and perform tests C and D to confirm lysogenic behavior.

C. Spontaneous phage release in liquid culture test (Supernatant assay)

Note: The estimated time to complete this section is 45 min of benchwork followed by overnight incubation.

1. Following purification of the colony of interest with two consecutive streak plates, inoculate the colony into a 10-mL Falcon tube containing ~4 mL LB.
2. Incubate overnight at 37°C with aeration.

Note: Aeration, achieved by leaving enough head space in the tube and incubating on a shaking platform at 150 rpm, is needed for optimal growth of the host used in this example. Growth conditions can be modified to cater to different hosts.

3. Centrifuge the culture at 3,500 × g for 10 min.
4. Collect the supernatant using a 10-mL syringe.
5. Attach a 0.2-µm filter to the syringe and carefully depress the plunger to filter the supernatant into a fresh tube.

Note: This step is optional. If not performed, we recommend repeating Steps C3 and C4.

6. Perform a spot assay (Procedure A), replacing the phage lysate with the filtered supernatant from C5.
7. For test interpretation, look for signs of lysis on the spot assay caused by the spontaneous release of phages from the lysogens into the supernatant of the liquid culture (Figure 1D).

D. Immunity assay

Note: The estimated time to complete this section is 15 min of benchwork followed by overnight incubation.

1. Perform a spot assay (Procedure A) replacing the original bacterial host with the candidate lysogen purified in Step B9.
2. For test interpretation, look for the absence of lysis caused by homoimmunity (no clearing, plaques, or loss of turbidity on the bacterial lawn) (Figure 1E).

Note: An immunity assay showing resistance to the original phage, without positive findings of lysogeny on Procedures B and C, is suggestive of alternative mechanisms of phage resistance. Conversely, a confirmed lysogen will test positive in Procedures B, C, and D. For an expanded explanation regarding interpretation of the procedures, see Figure 1 and Table 1.

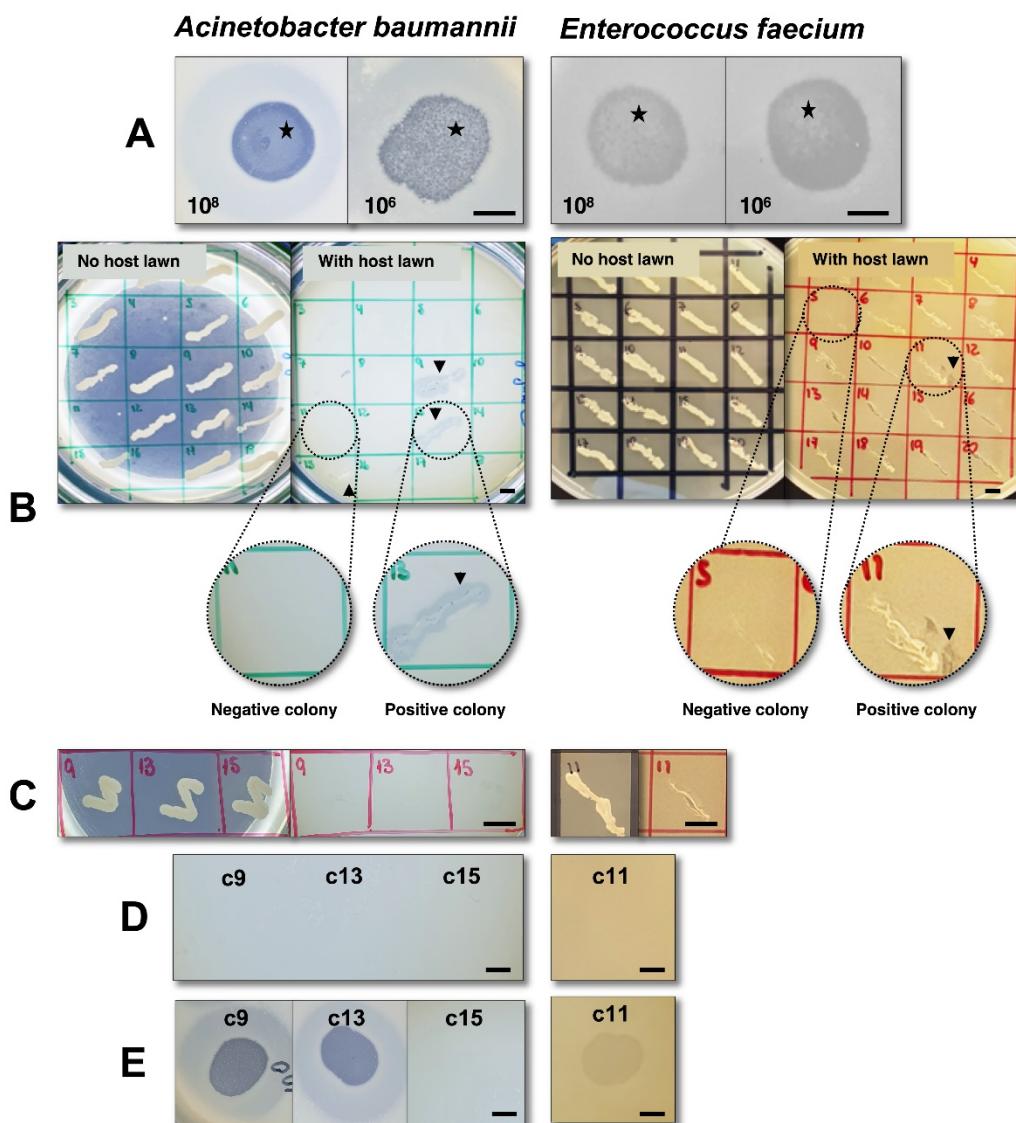


Figure 1. Screening for lysogenic behavior of phage φCO01 (Gordillo Altamirano *et al.*, 2021) on host strain *Acinetobacter baumannii* A9844 (Peleg *et al.*, 2008) and phage φFG-VRE (unpublished) on host strain *Enterococcus faecium* ST796 (Buultjens *et al.*, 2017).

A. Spot assay with droplets of 10⁸ and 10⁶ PFU/mL phage lysate after a 48-h incubation. Black stars denote mesas. B. First patch assay using plates with and without lawns of the bacterial hosts. Black arrows (colonies 9, 13, and 15 on *A. baumannii* and colony 11 on *E. faecium*) show bacterial growth with surrounding lysis, indicating the presence of phage, possibly released from a lysogen. Zoomed sections are provided as examples of positive and negative colonies. For *A. baumannii*, the panel only shows half the total screened colonies (18/36). C. Second patch plate, after two rounds of single-colony purification of colonies 9, 13, and 15 (*A. baumannii*) and colony 11 (*E. faecium*), without indication of phage presence when patched onto plates containing bacterial host lawns. D. Spot assay performed with the filtered supernatant from cultures of the putative lysogenic colonies, with no indication of spontaneous phage release. E. Immunity assay of colonies 9 and 13 (*A. baumannii*) and colony 11 (*E. faecium*) indicates sensitivity to their respective phages, thereby excluding homoimmunity; for colony 15 (*A. baumannii*), the test demonstrates resistance to the phage, most likely due to a mechanism other than homoimmunity (see Table 1 for interpretation). Taken together, the tests demonstrate the inability of phages φCO01 and φFG-VRE to lysogenize host strains *A. baumannii* A9844 and *E. faecium* ST796, respectively. For assays with *E. faecium*, LB was replaced with BHI (brain heart infusion) media. Scale bars = 0.5 cm.

Data analysis

A summary of the most frequent combinations of results in each of the four described procedures and a guide to their interpretation are included in Table 1. Briefly, lysogenic behavior of a phage can be safely excluded when at least 20 host colonies from at least two different mesas are negative in a patch test (Procedure B). Additionally, for a host colony to be confirmed as harboring the prophage, it must undergo double single-colony purification and subsequently test positive in Procedures B, C, and D.

Table 1. Interpretation guide. Most commonly encountered combinations of results and their interpretation.

Procedure Subject	A Mesa formation	B		C	D	Interpretation / Troubleshooting
		First patch test	Second patch test	Supernatant assay	Immunity assay	
Phage/host 1	-	N/A	N/A	N/A	N/A	Phage unable to lysogenize host (?) <i>Repeat A with lower phage titers and/or a longer incubation time</i>
Phage/host 2	+	-*	N/A	N/A	N/A	Phage unable to lysogenize host
Colony 1	N/A	+	-	-	-	Phage carried-over from the streak plate, no lysogen production
Colony 2	N/A	+	+	+	+	Successful lysogen production
Colony 3	N/A	-	-	-	+	Phage resistance due to other mechanisms
Colony 4	N/A	+	-	-	+	False positive on the first patch test, phage resistance due to other mechanisms

* At least 20 colonies screened, 10 from each of 2 different mesas. +: positive test. -: negative test.

Recipes

1. LB medium

10 g Bacto™ Tryptone
10 g granulated yeast extract
5 g NaCl
500 mL deionized water

Note: Autoclave, cool down, and store at room temperature.

2. LB agar plates

7.5 g agar powder
500 mL sterile LB medium

Note: Autoclave, allow to cool, pour ~20 mL per petri dish, allow to solidify, and store at 4°C.

3. LB top agar

1.5 g agar powder
200 mL sterile LB medium

Note: Autoclave, cool down, and store at room temperature. When needed, warm up in a microwave for ~3 min.

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We thank Prof Anton Y. Peleg and Prof Tim P. Stinear for providing the bacterial strains used in the present study. This protocol is derived from previous work by Broussard *et al.*, 2013 and Pope *et al.*, 2013 and is previously described in a summarized form by Gordillo Altamirano *et al.*, 2021.

Competing interests

The authors declare not to have any competing interests.

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COVID-19 Sample Pooling: From RNA Extraction to Quantitative Real-time RT-PCR

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Abstract

The COVID-19 pandemic requires mass screening to identify those infected for isolation and quarantine. Individually screening large populations for the novel pathogen, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), is costly and requires a lot of resources. Sample pooling methods improve the efficiency of mass screening and consume less reagents by increasing the capacity of testing and reducing the number of experiments performed, and are therefore especially suitable for under-developed countries with limited resources. Here, we propose a simple, reliable pooling strategy for COVID-19 testing using clinical nasopharyngeal (NP) and/or oropharyngeal (OP) swabs. The strategy includes the pooling of 10 NP/OP swabs for extraction and subsequent testing via quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), and may also be applied to the screening of other pathogens.

Keywords: COVID-19, Sample pooling, SARS-CoV-2, Pooling strategy, RNA extraction, RT-qPCR

This protocol was validated in: PLoS One (2020), DOI: 10.1371/journal.pone.0238417

Background

The coronavirus disease (COVID-19) pandemic has resulted in significant socioeconomic and public health burden in affected countries worldwide. The unprecedented spread of the disease has placed healthcare systems under considerable pressure, particularly in efforts to detect cases as efficiently as possible, contain the disease, and treat cases in a timely manner. Disease surveillance and the confirmation of positive COVID-19 cases has also mostly relied upon quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) of nasopharyngeal/oropharyngeal (NP/OP) swab specimens for the detection of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) following WHO guidelines (Corman *et al.*, 2020). However, the RT-qPCR testing capacity can be highly variable, particularly in rural areas, where primary healthcare facilities may not have trained personnel or the necessary equipment and facilities to carry out confirmatory testing. Hence, samples are sent to centralized facilities, which in turn are met with an overwhelmingly high demand for testing. As COVID-19 cases continue to rise, these laboratories have begun to face a backlog of pending tests and rising turnaround times that affect the clinical utility of results and timely surveillance of populations at the greatest risk.

In efforts to increase the cost-effectiveness and capacity of COVID-19 screening, we developed a pooling strategy during nucleic acid extraction without a reduction in the sensitivity of RT-qPCR (Lim *et al.*, 2020). The pooling of clinical samples has previously been employed for the screening of the human immunodeficiency virus (HIV), hepatitis C virus (HCV) (Hourfar *et al.*, 2008), influenza viruses (Pilcher *et al.*, 2005; Sullivan *et al.*, 2011), and during the COVID-19 pandemic, SARS-CoV-2 (Lim *et al.*, 2020). This method has been demonstrated to be cost-effective by allowing an increase in the viral detection capacity for mass screening while maintaining testing accuracy (Abdalhamid *et al.*, 2020). Pooled testing for RT-qPCR has been frequently utilized (Hogan *et al.*, 2020; Lohse *et al.*, 2020), unlike the strategy described in this paper, where we pool samples during nucleic acid extraction prior to performing RT-qPCR. This method has been evaluated as an effective approach in recent studies (Garg *et al.*, 2020; Wacharapluesadee *et al.*, 2020), where in a similar fashion, NP/OP swab samples were pooled in Viral Transport Medium (VTM) for extraction. Findings by Yelin *et al.*, (2020) suggest that even in pools of up to 32 samples, one positive sample could be detected.

Pooling during the extraction stage provides additional reductions in testing costs and workload. Using the strategy described in this protocol leads to a 9-fold reduction in the number of nucleic acid extractions and RT-qPCR reactions required (Lim *et al.*, 2020). For laboratories conducting screening for SARS-CoV-2, the pooled testing strategy used in this protocol does not introduce any significant changes to the current testing workflow and may be incorporated into routine procedures. We note that the optimal pool size will be dependent upon, among others, the prevalence of COVID-19, the objective of the testing strategy, and the resources available to the testing facility; therefore, it is recommended that laboratories account for these differences when adopting this strategy.

Thus, in this paper, we aim to provide a step-by-step protocol that details the pooling strategy for the screening of SARS-CoV-2. The strategy involves the pooling of 10 NP/OP swabs for extraction and subsequent testing by RT-qPCR. Implementing such a pooling strategy can minimize the costs incurred and the reagents utilized, while simultaneously reducing turnaround times for the rapid identification and isolation of positive COVID-19 cases. This protocol may also be applied to other pathogens that are screened using similar techniques, and would be particularly useful for the community screening of large groups in resource-poor settings and/or where the prevalence of infection is low.

Materials and Reagents

1. Personal protective equipment (PPE) appropriate for working with SARS-CoV-2
2. 0.2 mL low-profile PCR tube strips with caps (Bio-Rad, catalog numbers: TLS0851 and TCS0903)
3. 1.5 mL microcentrifuge tubes (Axygen, catalog number: MCT-150-C-S)
4. 0.5-10 µL, 0.5-50 µL, 1-200 µL, 100-1,000 µL filtered pipette tips (Axygen, catalog numbers: TF-400-R-S, TF-50-R-S, TF-200-R-S, TF-1005-WB-R-S)
5. Geneaid Viral Nucleic Acid Extraction Kit (Geneaid Biotech, catalog number: VR300)
6. Qiagen DNeasy Blood and Tissue Kit (Qiagen, catalog number: 69506)

7. SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, catalog number: 12574-026) (aliquot and store at -20°C)
8. DNase- and RNase-free water (Integrated DNA Technologies, catalog number: 11-05-01-04) (aliquot into clean 1.5-mL microcentrifuge tubes)
9. Primers for SARS-CoV-2 (Integrated DNA Technologies) (Table 1)

Table 1. Primers and probes targeting RNA-dependent RNA polymerase (RdRP), SARS-CoV-2 detection^a

Catalog number	Oligonucleotide	Sequence ^b
CV001	RdRp_SARSr-F	5'-GTGARATGGTCATGTGTGGCGG-3'
CV002	RdRp_SARSr-R	5'-CARATGTTAAASACACTATTAGCATA-3'
CV101	RdRP_SARSr-P1	5'-FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ-3'
CV102	RdRp_SARSr-P2	5'-FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ-3'

^a Oligonucleotide sequences were adapted from the WHO-Charité protocol (Corman *et al.*, 2020).

^b W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

10. Primer mix (see Recipes)

Equipment

1. 1 µL-10 µL, 10 µL-100 µL, 20 µL-200 µL, 100 µL-1,000 µL micropipettors (Eppendorf, catalog numbers: 3123000020, 3123000047, 3123000055, 3123000063)
2. CFX96 RealTime System (Bio-Rad, model: C1000 touch)
3. Thermo Aluminium Bath (Fine PCR, model: ALB128) to be used with the Qiagen DNeasy Blood and Tissue Kit
4. Centrifuge 5425 (Eppendorf, catalog number: 5405000417)

Procedure

All procedures in (A) sample pooling, (B) preparation of positive sample-pool, (C) ribonucleic acid (RNA) extraction, and (D) quantitative reverse transcription polymerase chain reaction (RT-qPCR) were carried out separately inside different biosafety cabinets. All clinical waste and consumables were autoclaved prior to being sent for incineration.

A. Sample pooling (see Figure 1)

1. We chose 10 sample-pools since the positive detection rate in our laboratory is less than 2%. You may optimize the sample-pool size according to the positive detection rate in your laboratory (Abdalhamid *et al.*, 2020).
2. Pipette 60 µL each of the 10 clinical NP/OP swabs into a single 1.5-mL microcentrifuge tube. The total volume is 600 µL. The original volume of the specimens ranged from 1 mL to 3 mL.
3. Aliquot 300 µL pooled swabs to each of two clean 1.5-mL microcentrifuge tubes and label with the same sample ID.

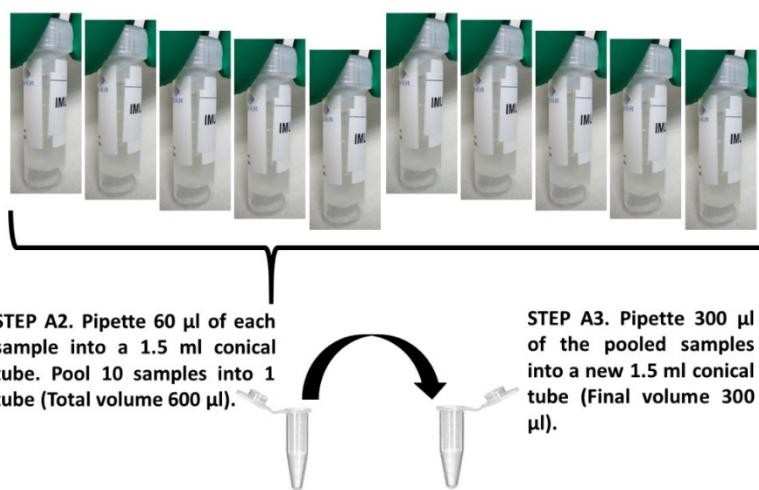


Figure 1. Pooling of 10 individual samples

B. Preparation of positive sample-pool

1. Identify a positive NP/OP swab that has a C_T value ≥ 15.00 but ≤ 36.00 (refer to Table S2 in Lim *et al.*, 2020).
2. Aliquot the identified positive NP/OP swab and store at -20°C .
3. Prepare the positive sample-pool according to Steps A2-A3 by mixing 1 positive with 9 unknown samples.

C. RNA extraction

1. Choose either of the kits below for RNA extraction
 - a. Geneaid Viral Nucleic Acid Extraction Kit
 - i. Add 400 µL VB Lysis Buffer to each of the 1.5-mL microcentrifuge tubes. Shake the tube vigorously for 10 s.
 - ii. Incubate at room temperature for 10 min.
 - iii. Add 450 µL AD Buffer (make sure that the ethanol has been added) to each of the 1.5-mL microcentrifuge tubes. Shake the tube vigorously to mix.
 - iv. Transfer 700 µL lysate mixture to the VB column in a 2-mL collection tube. Centrifuge at $14,000 \times g$ for 30 s. Discard the flowthrough and place the VB Column back into the 2-mL collection tube. Repeat Step (iv) until all the mixture (two 1.5-mL microcentrifuge tubes prepared in Step A3) has completely flowed through the VB column.
 - v. Add 400 µL W1 Buffer to the VB column and centrifuge at $14,000 \times g$ for 30 s. Discard the flowthrough and place the VB column back into the 2-mL collection tube.
 - vi. Add 600 µL Wash Buffer (make sure that the ethanol has been added) to the VB column. Centrifuge at $14,000 \times g$ for 30 s. Discard the flowthrough and place the VB column back into the 2-mL collection tube.
 - vii. Centrifuge at $14,000 \times g$ for 3 min to dry the column matrix.
 - viii. Place the dried VB column in a clean 1.5-mL microcentrifuge tube. Add 35 µL DNase RNase-free water to the center of the VB column matrix and incubate for 3 min at room temperature ($15\text{--}25^{\circ}\text{C}$).
 - ix. Centrifuge at $\geq 6,000 \times g$ for 1 min to elute the purified nucleic acid.
 - b. Qiagen DNeasy Blood and Tissue Kit
 - i. Pipette 20 µL Proteinase K into each 1.5-mL microcentrifuge tube.
 - ii. Add 200 µL Buffer AL and mix thoroughly by shaking vigorously.

- iii. Incubate at 56°C for 10 min.
- iv. Add 200 µL ethanol (96-100%) and mix thoroughly by shaking vigorously.
- v. Pipette 700 µL mixture into a DNeasy Mini spin column placed in a 2-mL collection tube. Centrifuge at \geq 6,000 \times g for 1 min. Repeat Step (v) until all the mixture (two 1.5-mL microcentrifuge tubes prepared in Step A3) has completely flowed through the spin column. Discard the flowthrough and collection tube.
- vi. Place the spin column into a clean 2-mL collection tube.
- vii. Add 500 µL Buffer AW1 and centrifuge for 1 min at \geq 6,000 \times g.
- viii. Discard the flowthrough and collection tube.
- ix. Place the spin column in a clean 2-mL collection tube, add 500 µL Buffer AW2, and centrifuge for 3 min at 14,000 \times g.
- x. Discard the flowthrough and collection tube.
- xi. Transfer the spin column into a clean 1.5-mL microcentrifuge tube.
- xii. Elute the RNA by adding 35 µL DNase- and RNase-free water to the center of the spin column membrane. Incubate for 3 min at room temperature (15-25°C).
- xiii. Centrifuge for 1 min at \geq 6,000 \times g to elute the purified nucleic acid.

D. RT-qPCR

1. Prepare the RdRP-Primer/Probe mixture (10 µM) (see Recipe).
2. Prepare 3 tests: (1) positive sample-pool; (2) non-template control (NTC); and (3) unknown sample-pool.
3. Prepare the RT-qPCR mixture using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase as described in Table 2.

Table 2. RT-qPCR mixture preparation

Reagent	Final concentration	Volume (µl) for one reaction
2× reaction buffer	1×	10.0
SuperScript™ III RT/Platinum™ Taq Mix	-	0.5
RdRP-Primer/Probe mixture (10 µM)	2 µM	1.0
Purified nucleic acid (10 sample pool) ^a	-	8.5
Total volume		20.0

^a Replace purified nucleic acid with DNase- and RNase-free water for NTC.

4. Run the RT-qPCR cycles as described below:
55°C for 15 min
95°C for 2 min
95°C for 15 s and 56°C for 30 s for 50 cycles

Data analysis

1. Interpret and record the results using the criteria below (see Notes for explanation):
 - a. If the C_T value is \leq 14.00, consider it as contamination. Repeat the RT-qPCR of the pooled purified nucleic acid. Upon detection of the same C_T value in the repeated RT-qPCR, deconvolute the positive pool(s) and repeat the nucleic acid extraction and RT-qPCR on individual samples from the positive pool(s). Test results are confirmed by a conventional RT-qPCR clinical test on a single sample.
 - b. If the C_T value is >14.01 but \leq 45, record the C_T value and consider positive detection of SARS-CoV-2 in

- the pooled samples. Deconvolute the positive pool(s) and repeat the nucleic acid extraction and RT-qPCR on individual samples from the positive pool(s). Test results are confirmed by a conventional RT-qPCR clinical test on a single sample.
- c. If the C_T value is ≥ 45.01 or no C_T value is detected, record the pooled samples as negative. Each single sample is reported as negative in this pool.
 2. An example of the analysis is shown in Tables 3, 4, and 5 (extracted Dataset S1 from Lim *et al.*, 2020). Here, we briefly describe the data shown in Tables 3-5.
- On 6th April 2020, the total number of specimens to be extracted was 101 (including an internal positive control). We performed sample pooling according to Steps A1-A3. Groups A-I were pools of 10, while Groups J and K were pools of 5 (total volume 300 μL) and 6 (total volume 360 μL), respectively. The RNA was extracted from the pooled samples as per Steps C1a.i-ix. The RT-qPCR was performed as per Steps D1-D4. Results of pooled testing are indicated in Table 3. SARS-CoV-2 was detected in Groups I and J. Individual specimens from both groups were individually extracted again according to the manufacturer's protocol and RT-qPCR was performed as per Steps D1-D4. The results of individual testing are stated in Tables 4 and 5 for Groups I and J, respectively.

Table 3. April 101th, 2020. IMU COVID-19 pooled testing results

No.	Group	Pooled samples (specimens), IMU Ref#	(10 Real-time RT-qPCR result of pooled samples (C_T value))	Proceed with individual testing
1	A	1276-1285	Not Detected	No
2	B	1286-1295	Not Detected	No
3	C	1296-1305	Not Detected	No
4	D	1306-1315	Not Detected	No
5	E	1316-1325	Not Detected	No
6	F	1327-1336	Not Detected	No
7	G	1337-1346	Not Detected	No
8	H	1347-1356	Not Detected	No
9	I ^a	1458-1466 (9 samples + <i>internal +ve control</i>)	COVID19 Detected (23.71)	Yes
10	J ^b	1467-1471	COVID19 Detected (40.16)	Yes
11	K ^b	1472-1477	Not Detected	No

^a**Group I** was prepared as per step B3, spiked with one internal positive control (9 samples + 1 **internal +ve control**).

^b**Groups J and K** were pools of 5 (total volume 300 μL) and 6 (total volume 360 μL), respectively. Aliquots of 150 μL (Group J) and 180 μL (Group K) were pipetted into two 1.5-mL microcentrifuge tubes as per step A3.

Table 4. Individual testing RT-qPCR results for pooled Group I

Group I	IMU Ref#	C_T value		
			Pooled testing (1 st qRT-PCR)	Individual (2 nd qRT-PCR)
1	1458			Not Detected
2	1459			Not Detected
3	1460			Not Detected

4	1461	COVID19 Detected (23.71)	Not Detected
5	1462		Not Detected
6	1463		Not Detected
7	1464		Not Detected
8	1465		Not Detected
9	1466		Not Detected
10	<i>Internal +ve control</i>		COVID19 Detected (23.23)

Table 5. Individual testing RT-qPCR results for pooled Group J

Group J	IMU Ref#	C _T value	
		Pooled testing (1 st RT-qPCR)	Individual (2 nd RT-qPCR)
1	1467		Not Detected
2	1468		Not Detected
3	1469	COVID19 Detected (40.16)	Not Detected
4	1470		Not Detected
5	1471		Not Detected

Notes

C_T value: We chose the C_T value cut-off based on our preliminary data obtained by optimizing the pooling. We noticed that a weak positive C_T value of 38 may increase to 40 after pooling; thus, we recommend a C_T value cut-off of 45 to safeguard the accuracy and precision of the detection method. Any pooled samples considered to be positive, with C_T values detected below 45, will need to be individually tested for accurate reporting. Please see the example provided in Table 3.

Recipes

1. Primer mix

- Prepare each one of the primers and probes at 100 μM
- Primer/probe mix (100 μL):
 - RdRp_SARSr-F 10 μL (10 μM final concentration)
 - RdRp_SARSr-R 10 μL (10 μM final concentration)
 - RdRP_SARSr-P1 10 μL (10 μM final concentration)
 - RdRp_SARSr-P2 10 μL (10 μM final concentration)
- DNase- and RNase-free water 60 μL
- Aliquot and store at -20°C

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support in sharing the necessary reagents for laboratory testing. This protocol was derived from our previous work (Lim *et al.*, 2020).

Competing interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. While performing this research, no company had invested in the research and no commercialization was intended.

Ethics

This study obtained ethical clearance from the International Medical University Joint Research and Ethical Committee (EC/IRB Ref. No. 4.41/JCM-196/2020). All clinical specimens used in this study were fully anonymized and deidentified by assigning new laboratory reference numbers before access was given to the researchers. The researchers were blinded to patient information.

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Histological Methods to Detect Early-stage Plant Defense Responses during Artificial Inoculation of *Lolium perenne* with *Epichloë festucae*

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Abstract

Epichloë species form agriculturally important symbioses with many cool season grasses. To study these symbioses, such as the interaction of *Epichloë festucae* with perennial ryegrass (*Lolium perenne*), host plants can be infected by artificial inoculation of etiolated seedlings. This inoculation is performed by placing mycelium into an incision in the meristem, as previously described by Latch and Christensen (1985). In recent years, this method has been broadly used to study this interaction at the molecular level using different *Epichloë festucae* mutants that can cause incompatible interactions. We have developed and adapted methods to study four of the most important host plant responses to infection, including cell death, callose deposition, lignin production, and hydrogen peroxide (H_2O_2) production, which are useful in defining the host response to infection at a very early time point.

Keywords: Endophytic fungi, Plant defense, Mutualism, Symbiotic, Plant-fungal interactions, ROS production

This protocol was validated in: Mol Plant Microbe Interact. (2018) DOI: 10.1094/MPMI-11-17-0286-R.

Background

Although artificial inoculation of perennial ryegrass (*Lolium perenne*) is broadly used to study its interaction with *Epichloë festucae*, there exists no comprehensive protocol for the evaluation of responses to fungal infection at an early post-inoculation time point, which likely defines the future of this interaction. In our recent work (Rahnama *et al.*, 2018), we adapted methods to study four of the most important host plant responses during plant-fungal interactions including cell death, callose deposition, lignin production, and hydrogen peroxide (H₂O₂) production. In response to invading microbes, one of the early plant actions is the production of different types of reactive oxygen species (ROS), including H₂O₂, which act as an antimicrobial agent to protect the plant against invading microbes and are one of the first signals to induce other plant responses (Walters, 2003). In addition, by depositing callose and lignin, plants remodel their cell wall at the infection site to stop invading fungi (Luna Diez *et al.*, 2010; Zipfel, 2009). As a late defense response, plant cells surrounding the infection site undergo programmed cell death (hypersensitive response) to limit available food for the invading microbe (Lo Presti *et al.*, 2015). To initiate and maintain a successful symbiotic lifestyle, *Epichloë* endophytes must somehow suppress or avoid these different host defense responses; although, very little is known about how this occurs. Developing methods to detect these responses will aid in the understanding of this system, especially when applied to the study of mutant strains that compromise symbiosis.

To stain for callose deposition, we adapted an Aniline Blue staining method (Knox, 1979) that was originally used for the detection of callose in pollen tubes but has also been used in different fungal-plant interactions such as powdery mildew infection of *Arabidopsis* (Ellinger *et al.*, 2013). Besides aniline blue, Toluidine Blue O has also been used in other systems such as guava root infection with *Fusarium* (Gupta *et al.*, 2012). In addition to staining methods, there exist non-staining methods using immunofluorescence that detect callose at the micro-level in internal cell sites such as plasmodesmata (Pendle and Benitez-Alfonso, 2015).

To detect lignin deposition, we used a Safranin solution adapted from a study on maize infection by *Ustilago maydis* (Tanaka *et al.*, 2014); however, Safranin is broadly used in other systems such as *Fusarium* infection of different cereals (Knight *et al.*, 2011). Moreover, the Wiesner (phloroglucinol-HCl) reaction (Pomar *et al.*, 2002) is another method to study lignin deposition in plant-fungal interactions.

To visualize cell death responses, Trypan Blue staining is the most widely used method, for which we adapted the protocol used to study *Arabidopsis* infection with downy mildew (Koch and Slusarenko, 1990). Recently, a non-toxic method has also been suggested, which uses red light imaging (Landeo Villanueva *et al.*, 2021).

There are several methods to detect H₂O₂, one of the basic methods for which is the detection of the oxidization of small molecules, which we also used here. This method is based on visualizing the color change of 3,3'-diaminobenzidine (DAB) after being oxidized by H₂O₂. We adapted this method from a study on barley infection with powdery mildew (Thordal-Christensen *et al.*, 1997). Other recent methods to study H₂O₂ have used genetically encoded green fluorescent protein (GFP)-based probes that react directly with H₂O₂ (reviewed in Winterbourn, 2018).

Here, we demonstrate that these methods are useful for defining the response of perennial ryegrass to infection with *Epichloë* endophyte at a very early time point. Using these methods in time-point studies, we defined the most suitable time to measure these responses post-inoculation. As such, these methods are useful for studying compatible and incompatible interactions of symbiotic fungi with their host plants during the early stages of infection (Rahnama *et al.*, 2018 and 2019). Although these methods are routinely used for other fungal-plant interactions, our adaption of them to study the early inoculation stages of *Epichloë*-grass interactions is novel. These methods can be used in other grass-endophyte interactions that use a similar inoculation technique.

Materials and Reagents

1. 1.5 mL Pierce microcentrifuge tubes (Thermo Scientific, catalog number: 69715)
2. Petri dishes (Fisher brand, Fisher Scientific, catalog number: FB0875712)
3. Microscope slides (Pearl, catalog number: 7101)
4. Microscope slide coverslips (Pearl)

5. 1 mL Sterilin plastic transfer pipettes (Thermo Scientific, catalog number: 201C)
6. Surgical blade No.11 for metal scalpel 10621 (Feather, catalog number: 2976)
7. Household aluminum foil
8. Root trainers (Flight Plastic Ltd.)
9. Grass seeds (used here: *Lolium perenne* cv Samson seeds, endophyte-free; Agricom, New Zealand)
10. An *Epichloë* strain, *E. festucae* Fl1 (ATCC, catalog number: MYA-3407)
11. Autoclaved distilled water
12. Technical grade agar (Difco Laboratories, catalog number: DF0812-17-9)
13. Potato dextrose broth (Difco Laboratories, catalog number: DF0549-17-9)
14. L-lactate hydrate ($C_3H_6O_3$) (Sigma-Aldrich, catalog number: L1750)
15. Glycerol ($HOCH_2CH(OH)CH_2OH$) (Sigma-Aldrich, catalog number: G9012)
16. Phenol (C_6H_5OH) (Thermo Scientific, catalog number: 17914)
17. Trypan Blue ($C_{34}H_{24}N_6O_{14}S_4Na_4$) (Sigma-Aldrich, catalog number: T6146)
18. Chloral hydrate ($Cl_3CCH(OH)_2$) (Sigma-Aldrich, catalog number: C8383)
19. Aniline Blue W.S. ($C_{32}H_{25}N_3Na_2O_9S_3$) (Sigma-Aldrich, catalog number: 28631-66-5)
20. Tripotassium orthophosphate (K_3PO_4) (VWR, catalog number: 700001)
21. Safranin O (C.I. 50240) ($C_{20}H_{19}ClN_4$) (Sigma-Aldrich, catalog number: 1159480025)
22. 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich, catalog number: D12384)
23. Hydrochloric acid (HCL) (Sigma-Aldrich, catalog number: D12384)
24. 95% Ethanol (CH_3CH_2OH) (Sigma-Aldrich, catalog number: 11727)
25. Sucrose (Sigma-Aldrich, catalog number: S8501)
26. Boric acid (BH_3O_3) (Thermo Scientific, catalog number: AC315185000)
27. 2.4% Potato dextrose agar (see Recipes)
28. 3% Water agar (see Recipes)
29. Lactophenol-Trypan Blue solution (see Recipes)
30. Chloral hydrate solution (see Recipes)
31. Aniline Blue solution (see Recipes)
32. Pollen germination slides (see Recipes)
33. Safranin solution (see Recipes)
34. DAB solution (see Recipes)

Equipment

1. 100 mL glass beaker
2. Leica DMR microscope (camera: Leica DC500)
3. Autoclave
4. Fridge
5. Laminar flow cabinet
6. Incubator (Thermo Scientific, 3110 CO₂ Water-Jacketed Incubator)
7. Stereomicroscope (Leica, model: Leica M3Z)
8. Precellys 24 tissue disruptor (Bertin Technologies)
9. Stainless-steel forceps (Sigma-Aldrich, catalog number: Z168777)
10. Metal scalpel (Sigma-Aldrich, catalog number: S2646)

Procedure

A. Seedling preparation and inoculation (adapted from Latch and Christensen, 1985):

1. Seed surface sterilization:

- a. Incubate endophyte-free seeds in 50% sulfuric acid (H_2SO_4) for 30 min.
 - b. Pour off the acid and soak the seeds 3 times in sterile water for 3 min each time.
 - c. Incubate the seeds in 50% commercial bleach for 20 min.
 - d. Pour off and soak the seeds 3 times in sterile water for 2 min each time.
 - e. Dry the seeds on sterile filter paper in a laminar flow hood.
2. Seed culture:
 - a. Using sterile forceps, transfer the sterile dried seeds to 4% water agar (10 seeds per plate).
 - b. Incubate the plates in the dark at 22°C for 7 days.
 3. Mycelium preparation for inoculation:
 - a. Subculture the *Epichloë* strain onto a PDA plate.
 - b. Incubate the plate at 20°C for 7-10 days.
 4. Seedling inoculation:
 - a. After 7-8 days, the seeds will germinate with etiolated shoots.
 - b. Using a dissecting microscope in a laminar flow hood, make a small incision in the meristem (usually appears as a faintly visible line between the mesocotyl and the coleoptile) with a scalpel.
 - c. Cut a small piece of fungal mycelium from the PDA plate (around 2 mm × 2 mm) and insert into the incision in the meristem.
 - d. Incubate the seedlings in their original water agar plates at 22°C in the dark, with the plates standing on their end and the seedlings upright.
 - e. Subsequent steps are described in detail (including videos and figures) in Becker *et al.* (2018).

In all plant response tests, there are 4 inoculation treatments including seedlings with an incision inoculated with wild type *E. festucae* ("Wild Type"), mutant *ΔvelA* *E. festucae* ("ΔvelA"), a block of agar without fungi ("E- (cut)"), and seedlings without an incision but a piece of agar placed over the meristem ("E- (Uncut)").

B. Staining for defense responses

Inoculated seedlings were incubated for the different plant response tests as follows:

1. Callose deposition (adapted from Knox, 1979):
 - a. *Impatiens walleriana* pollen tubes can be used as a positive control to test whether the Aniline Blue solution is working properly.
 - i. Sprinkle pollen over a freshly prepared pollen germination slide.
 - ii. Store the slides in a moist Petri dish (containing wet tissue paper) for at least 5 h until germination occurs.
 - iii. Cover the slide with a few drops of Aniline Blue solution and incubate at room temperature for 30 min.
 - iv. Rinse the slides twice with distilled water.
 - v. Observe callose deposition in the pollen tubes using a fluorescence microscope (excitation 450-490 nm, emission >515 nm).
 - vi. Callose deposition appears as a yellow-green color (Figure 1A).
 - b. Studying callose deposition at different time points determined that 4 days post-inoculation (DPI) was optimal for *Epichloë*-ryegrass associations.
 - i. At 4 DPI, place the seedlings in Aniline Blue solution for 30 min at room temperature.
 - ii. Rinse the seedlings twice with distilled water.
 - iii. Remove the inoculum (fungal block, or agar block in the negative controls) from the meristem and cut out the meristem section of the seedlings including the 1.5 cm above and below.
 - iv. Observe callose deposition under a fluorescence microscope (excitation 450-490 nm, emission >515 nm).
 - v. Callose deposition appears as a yellow-green color (Figure 1B).

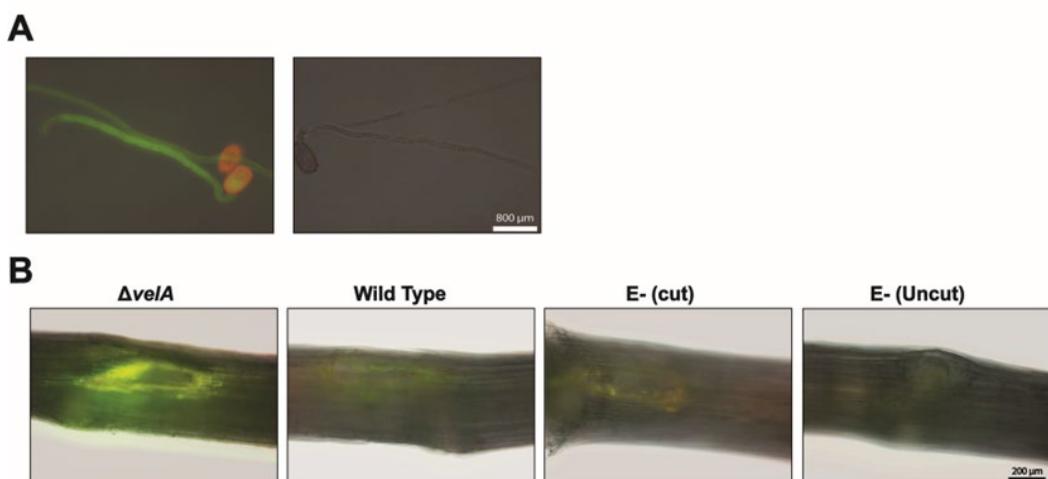


Figure 1. Callose detection.

A. Using *Impatiens walleriana* pollen tubes as a positive control for callose detection. Left panel; pollen tubes in Aniline Blue solution, right panel; pollen tubes in only buffer without Aniline Blue. B. Callose deposition in the meristematic region of 7-d-old seedlings inoculated with wild type and $\Delta velA$ mutant strains of *E. festucae* and endophyte-free cut and uncut seedlings inoculated with agar block as a control; 4 DPI stained with Aniline Blue solution. Callose deposition appears yellow-green.

2. Lignin production (adapted from Tanaka *et al.*, 2014):

Studying lignin production at different time points determined that 2.5 DPI was optimal for *Epichloë*-ryegrass associations.

- a. At 2.5 DPI, place the seedlings in Safranin solution for 10 min in the dark at room temperature.
- b. Rinse the seedlings twice with distilled water
- c. Remove the inoculum (fungal block, or agar block in the negative controls) from the meristem and cut out the meristem section of the seedlings including the 1.5 cm above and below.
- d. Observe lignin under a fluorescence microscope in brightfield.
- e. Lignin deposition appears as a red color (Figure 2).

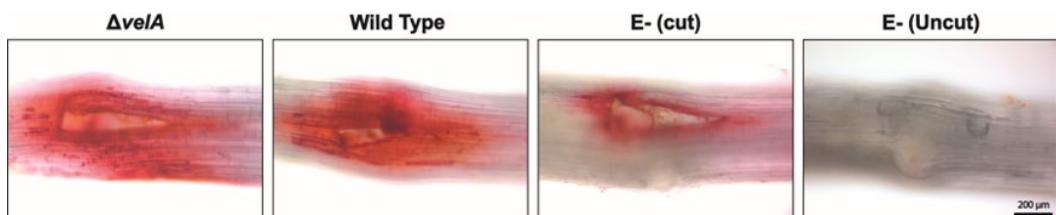


Figure 2. Lignin detection in the meristematic region of 7-d-old seedlings inoculated with wild type and $\Delta velA$ mutant strains of *E. festucae* and endophyte-free cut and uncut seedlings inoculated with agar block as a control; 4 DPI stained with 1% Safranin O solution.

Lignin deposition appears red.

3. Plant cell death (adapted from Koch and Slusarenko, 1990):

- a. The highest levels of plant cell death were observed at 7 days post-inoculation (DPI), but a time-course study can be performed from 1-12 DPI.
- b. At 7 DPI, place whole seedlings in 20 mL boiling lactophenol-Trypan Blue for 1 min. Since this solution contains corrosive phenol and requires boiling, this step should be carried out under the laminar flow cabinet. First, warm the solution in a 100-mL glass beaker over a Bunsen burner until boiling, then add the seedlings.

- c. Decolorize the stained seedlings by placing in 20 mL chloral hydrate solution for 30 min. Since chloral hydrate is toxic, this step should be carried out under the laminar flow cabinet.
- d. Rinse the seedlings twice with distilled water.
- e. Remove the inoculum (fungal block or agar block in the negative controls) from the meristem and cut out the meristem section of the seedlings including the 1.5 cm above and below.
- f. Mount the seedling sections on slides with coverslips in water.
- g. Observe the cell death response under a Nikon Ti-E inverted microscope (camera: Nikon DsRi1) in brightfield.
- h. Dead cells appear dark blue (Figure 3).

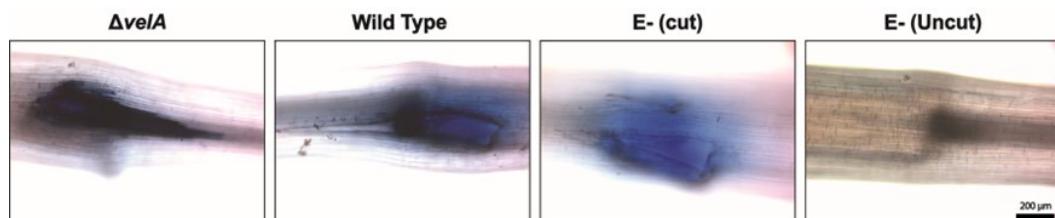


Figure 3. Cell death detection in the meristematic region of 7-d-old seedlings inoculated with wild type and $\Delta velA$ mutant strains of *E. festucae* and endophyte-free cut and uncut seedlings inoculated with agar block as a control; 4 DPI stained with lactophenol-Trypan Blue solution.
Dead cells appear dark blue.

4. Hydrogen peroxide (H_2O_2) production (adapted from Thordal-Christensen *et al.*, 1997):
 - a. Detecting H_2O_2 production after the normal inoculation procedure
 - i. Most H_2O_2 production should be detected immediately after inoculation.
 - ii. Immediately after inoculation, place whole seedlings in freshly prepared DAB solution for 4 h in the dark at room temperature.
 - iii. Rinse the seedlings twice with distilled water
 - iv. Remove the inoculum (fungal block, or agar block in the negative controls) from the meristem and cut out the meristem section of the seedlings including the 1.5 cm above and below.
 - v. Observe H_2O_2 production under a Leica DMR microscope in brightfield.
 - vi. H_2O_2 appears as a brown color (Figure 4A).
 - b. Detecting H_2O_2 production without incision
Since cutting the meristem produces a high level of H_2O_2 , making the differentiation between treatments difficult, we optimized the methodology to measure H_2O_2 in the absence of an incision.
 - i. Place 1-2 mm² fungal culture on agar blocks 1 cm above the meristem.
 - ii. Immediately after inoculation, place whole seedlings in freshly prepared DAB solution for 4 h in the dark at room temperature.
 - iii. Remove the inoculum (fungal block, or agar block in the negative controls) from the meristem and cut out the meristem section of the seedlings including the 1.5 cm above and below.
 - iv. Observe H_2O_2 production under a Leica DMR microscope in brightfield.
 - v. H_2O_2 appears as a brown color (Figure 4B).

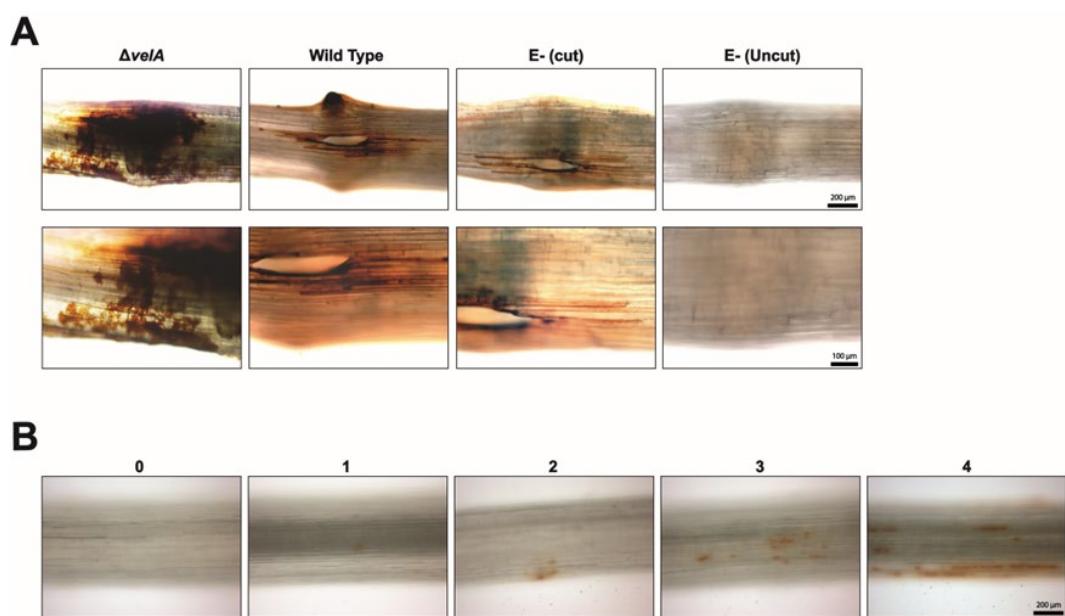


Figure 4. Hydrogen peroxide (H_2O_2) detection.

A. H_2O_2 production in the meristematic region of 7-d-old seedlings inoculated with wild type and $\Delta velA$ mutant strains of *E. festucae* and endophyte-free cut and uncut seedlings inoculated with agar block as a control; immediately stained with DAB solution. B. H_2O_2 response 1 cm above the meristem of 7-d-old seedlings was detected immediately after inoculating seedlings using method 4b (Detecting H_2O_2 production without incision). Arbitrary scoring system for measuring the severity of the response. H_2O_2 appears brown.

Data analysis

For all detection methods and for each treatment, a range of responses can be observed because ryegrass is an out-crossing species; therefore, each plant has a different genotype. As such, we used an arbitrary categorization method based on the percentage of seedlings that show the response. For all the methods (except 4b; Detecting H_2O_2 production without incision), data analyses are based on the percentage of seedlings showing the test response (similar to Figure 1B, first on the left). As an example, the results of callose deposition in seedlings inoculated with different strains of *E. festucae* are presented in Figure 5.

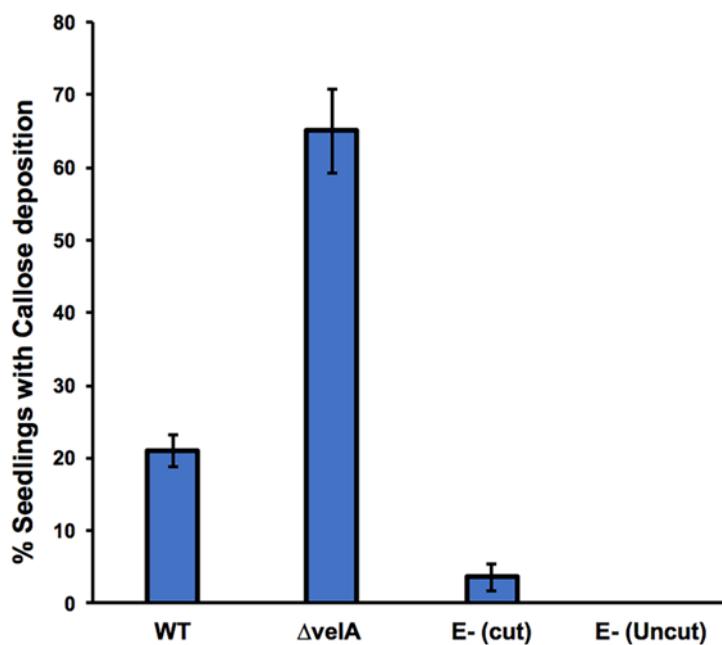


Figure 5. Percentage of 7-d-old seedlings showing callose deposition at 4 days post-inoculation.

Seedlings were inoculated with wild type and $\Delta velA$ mutant strains of *E. festucae*, and endophyte-free cut and uncut seedlings were inoculated with agar block as a control. In each inoculation, 30 seedlings were inoculated. Bars represent the standard error of the mean calculated from 3 independent inoculation experiments.

To detect H₂O₂ production without incision (method 4b), a range of 5 different responses (0 to 4) was used (Figure 4B) due to the sensitivity of the method. The percentage of seedlings showing an H₂O₂ response can be visualized in Figure 6.

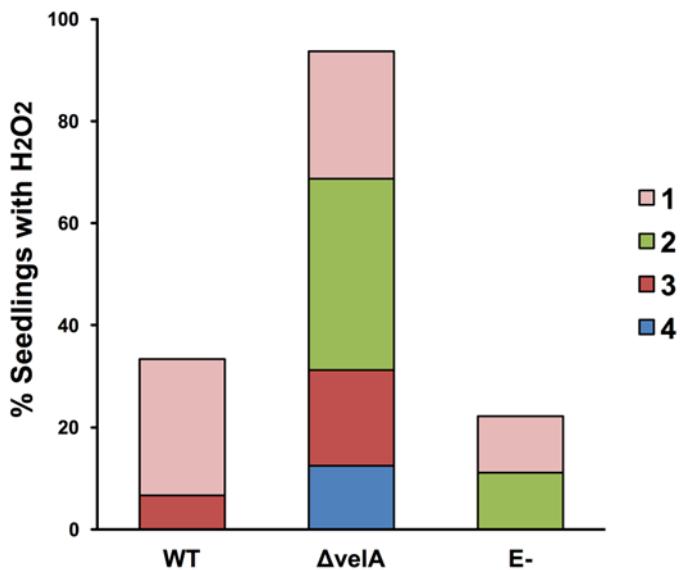


Figure 6. Percentage of 7-d-old seedlings showing the production of hydrogen peroxide (H₂O₂).

The H₂O₂ response 1-cm above the meristem of 7-d-old seedlings was detected immediately after inoculating seedlings using method 4b (detecting H₂O₂ production without incision) with wild type (WT) and $\Delta velA$ mutant strains of *E. festucae* and agar block as a control (E-). In each inoculation, 30 seedlings were inoculated.

Numbers 1-4 in the legend represent arbitrary categorization of the H₂O₂ responses represented in Figure 4B.

Notes

1. Three independent inoculation tests were performed for each method.
2. The number of seedlings used for each test should be greater than 30, and increasing the number of seedlings can help to reduce the variability of the responses that can result from differences in genetic background of the seeds.
3. In all the detection methods, we normally stain whole seedlings and cut out the meristem region (region of interest) for microscopy. This reduces host responses due to wounding that can interfere with microscopy.

Recipes

1. 2.4% potato dextrose agar

- a. Dissolve 24 g potato dextrose broth and 15 g agar in 1 L distilled water
- b. Autoclave for 30 min at 121°C and pour 25 mL solution per Petri dish
- c. Plates can be stored in the fridge for up to one month

2. 3% water agar

- a. Dissolve 30 g agar in 1 L distilled water
- b. Autoclave for 30 min at 121°C and pour 25 mL solution per Petri dish
- c. Plates can be stored in the fridge for up to one month

3. Lactophenol-trypan blue solution

- a. Dissolve 10 mg trypan blue in 10 mL distilled water and add 10 mL lactic acid (98%), 10 mL glycerol, and 10 mL phenol
- b. The solution can be stored in the dark in the fridge for up to 3 months

4. Chloral hydrate solution

- a. Dissolve 2.5 g chloral hydrate in 1 mL distilled water
- b. The solution can be stored in the fridge for up to 1 month

5. Aniline blue solution

- a. Dissolve 0.1 g aniline blue and 2.3 g tripotassium orthophosphate in 100 mL distilled water
- b. Place the solution in the dark for 24 h until it becomes colorless
- c. The solution can be stored in the dark in the fridge for up to 3 months

6. Pollen germination slides

- a. Dissolve 10 g sucrose, 1 g agar, and 8 mg boric acid in 100 mL distilled water
- b. Autoclave for 30 min at 121°C and drop on a slide to make a flat film
- c. Slides can be stored in the fridge for up to one week

7. Safranin solution

- a. Dissolve 1 g Safranin O (C.I. 50240) in 100 mL distilled water
- b. The solution can be stored in the dark in the fridge for up to 3 months

8. DAB solution

- a. Dissolve 10 mg 3,3'-diaminobenzidine (DAB) in 10 mL distilled water

- b. Adjust the pH to 3.8 using HCl
- c. The solution should be prepared fresh for each test

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Competing interests

The authors declare no conflicts of interest.

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A Fluorescence Dequenching-based Liposome Leakage Assay to Measure Membrane Permeabilization by Pore-forming Proteins

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Abstract

Pore-forming toxins (PFTs) have been discovered in a wide range of organisms. Their functions are essential to the survival or virulence of many species. PFTs often interact with lipid membranes. Large unilamellar vesicles (LUV), also known as liposomes, have been commonly used as reliable membrane models for testing PFTs activity. Liposomes have great adaptability in size, lipid composition, and loading cargo. Incorporating the fluorescent dye/quencher pair, 8-Aminonaphthalene-1,3,6-Trisulfonic Acid (ANTS) and p-Xylene-Bis-Pyridinium Bromide (DPX), in liposomes is an effective approach for measuring membrane leakage. When ANTS and DPX are encapsulated in a liposome, the fluorescence of ANTS is quenched by DPX. However, disruption of liposome integrity and subsequent leakage result in measurable fluorescence emitted by ANTS. Here, we report our protocol for optimal liposome preparation for measuring liposome leakage by fluorescence dequenching.

Keywords: Fluorescence dequenching, Liposome leakage, ANTS, DPX, EsxA, EsxB, *Mycobacterium tuberculosis*

This protocol was validated in: J. Biol. Chem. (2020) DOI: 10.1074/jbc.RA119.012497

Background

Pore-forming toxins (PFTs) are a family of virulence factors produced by microbial pathogens for host invasion (Alouf *et al.*, 2005). The major action of PFTs is forming pores on lipid membranes, resulting in membrane lysis and/or translocation of other effector proteins into host cells (Bischofberger *et al.*, 2009). Liposomes have long been used as a biological membrane model for protein-membrane interactions due to their great adaptability in size, lipid composition, and loading cargo (Chatterjee and Agarwal, 1988). A common way of using liposomes to measure pore formation or membrane leakage is to encapsulate ions or fluorescent dyes inside liposomes. Upon liposome rupture, the released ions or fluorescent dyes emit measurable signals. We first used a K⁺ release assay to measure pore formation on liposomal membranes by the anthrax toxin (Sun *et al.*, 2007 and 2008; Sun and Collier, 2010). Later, the fluorescence dye and quencher pair ANTS/DPX caught our attention (Nieva *et al.*, 1989; Ruiz-Argüello *et al.*, 1996). Compared with the K⁺ release assay, which requires a bulky K⁺ probe and frequent changes of costly probe membranes, the ANTS/DPX dequenching assay featured higher sensitivity, more accurate and consistent measurements, and lower cost. Therefore, over the past ten years, we have transitioned to the ANTS/DPX dequenching assay. With this assay, we have successfully measured the membrane permeabilizing activity (MPA) of *Mycobacterium tuberculosis* (Mtb) virulence factors. The two effector proteins, 6-kDa early secreted antigenic target (ESAT-6) or EsxA and 10-kDa culture filtrate protein (CFP-10) or EsxB have been tested with various experimental designs and settings (De Leon *et al.*, 2012; Ma *et al.*, 2015; Peng *et al.*, 2016; Zhang *et al.*, 2016; Aguilera *et al.*, 2020; Ray *et al.*, 2019). EsxAB is a heterodimer implicated in penetrating the phagosomal membrane of macrophages, allowing for translocation of Mtb into the cytosol (De Leon *et al.*, 2012; Ma *et al.*, 2015; Zhang *et al.*, 2016; Aguilera *et al.*, 2020). Recently, we have identified a new lipid composition for liposomes that greatly enhances the MPA of EsxA (Ray *et al.*, 2019; Vazquez-Reyes *et al.*, 2020). Here, we describe in detail the fluorescence dequenching based liposome leakage assay with our new lipid composition.

Materials and Reagents

1. Screw cap glass vials with PTFE screwcap (Spectrum Chemical, catalog number: 985-92215)
2. Cuvette spinbar (VWR, catalog number: 58949-030)
3. Quartz UV cuvette (FireflySci, catalog number: 1FLUV10)
4. 515 nm filter (Andover Corporation, catalog number: 515FG05-50S)
5. 1 mL syringe (BD, catalog number: 309659)
6. 9" Pasteur pipet (Fisher Scientific, catalog number 13-678-6b)
7. Saint-Gobain Tygon tubing (Saint-Gobain, catalog number: ACF00017F)
8. Glass tube rack (Fisher Scientific, catalog number: K7492100100)
9. HiTrap® desalting columns (Millipore-Sigma, catalog number: GE17-1408-01)
10. Gel loading pipet tips (Fisherbrand, catalog number: 02-707-139)
11. 10 mm filter supports (Avanti Polar Lipids, catalog number: 610014)
12. 0.2 µm membrane (Whatman, catalog number: 800281)
13. Aluminum foil (Fisherbrand, catalog number: 01-213-100)
14. 1 mL sample loop (Thermo Scientific, catalog number: 03-052-382)
15. Chloroform (Fisher Chemical, catalog number: c298)
16. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, catalog number: 850375)
17. 1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl] (nickel salt) (18:1 DGS-NTA(Ni)) (Avanti Polar Lipids, catalog number: 790404)
18. 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) (Avanti Polar Lipids, catalog number: 840457)
19. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, catalog number: 850457)
20. Ethanol (Fisher Chemical, catalog number: a4094)
21. HPLC grade water (Fisher Chemical, catalog number: W5-4)
22. Tris-base (Fisher Chemical, catalog number: BP152)

23. Sodium chloride (Fisher Chemical, catalog number: BP358)
24. Sodium hydroxide (Fisher Chemical, catalog number: S318)
25. Hydrochloric acid (Fluka, catalog number: 7647)
26. Dry ice
27. 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Fisher Bioreagents, catalog number: BP310)
28. 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (Setareh Biotech, catalog number: 6951)
29. p-Xylene-bis-pyridinium bromide (DPX) (Setareh Biotech, catalog number: 6271)
30. Triton X-100 (Sigma-Aldrich, catalog number: X100)
31. Sodium acetate (Sigma-Aldrich, catalog number: S2889)
32. Methanol (Fisher, catalog number: A453-500)
33. Gel filtration buffer (see Recipes)
34. pH 4 buffer (see Recipes)

Equipment

1. Glass tubes (VWR, catalog number: 89000-502)
2. 50 mL beaker (Pyrex, catalog number: 02-540G)
3. Lab mounting stand (Humboldt MFG Company, catalog number: H-21217)
4. Nitrogen gas tank
5. Gas tank regulator (VWR, catalog number: 55850-277)
6. Large top desiccator (Corning Life Sciences, catalog number: CLS3120250)
7. Hot plate/stirrer (Thermo Fisher Scientific, catalog number: SP131325)
8. Extruder set with heating block (Avanti Polar Lipids, catalog number: 610000)
9. pH meter (Mettler Toledo, catalog number: 30266626)
10. Vortex (VWR, catalog number: 58816-121)
11. Scale (Mettler Toledo, catalog number: ML54)
12. AKTA FPLC system (General Electric, catalog number: UPC-900 and P920)
13. Fluorometer (ISS, catalog number: K2)
14. Water bath (VWR, catalog number: 13271-086)

Procedure

A. Drying phospholipids

1. If working with lipid powders, create a lipid stock solution.
2. Measure 50-150 mg of lipids into a glass vial with a screw cap.
3. Add 5-15 ml of 3:2 chloroform:methanol as needed to maintain concentration at 10 mg/mL for ease of usage.

Note: Cover the vial using PTFE lined caps; otherwise, the chloroform may dissolve plastics and contaminate the sample.

4. Measure the appropriate amount of lipids (10 mg will be enough for approximately 30 reactions) into a glass tube.

Note: Liposomes can be made of single lipid forms [e.g., 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)] or their combinations. For example, the Ni²⁺-

chelating lipid, 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) or (DGS-NTA(Ni)), can attach His-tagged proteins to the membranes (Sun et al., 2007 and 2008; Jacquez et al., 2014). The new liposome developed for measuring the MPA of EsxAB is composed of POPC to POPG at a 4:1 molar ratio.

5. Use a clamp stand to hold the vial in place.
6. Using a second clamp, position a Pasteur pipette approximately 2-5 mm away from the liquid in the tube.
7. Connect the tubing to the end of the Pasteur pipette and open the valve to allow gentle airflow.
8. Evaporate the chloroform from the vial until there is no liquid visible.
9. Remove the vial from the stand and place it inside a vacuum chamber at room temperature for at least 3 h or overnight.
10. Store the dry lipids at -20°C. Lipids may be stored under these conditions for up to a month.

B. Preparing column and FPLC for desalting

1. A 5-mL desalting column is needed for removing the excess ANTS/DPX and for buffer exchange. If necessary, two desalting columns can be connected in tandem to improve desalting results.
2. Pass at least 5 column volumes (CV) or, in this case, 50 mL of water through the column to remove the ethanol from the column.
3. Pass at least 5 CV (50 mL) of gel filtration buffer through the column.
4. Prepare the machine for desalting by passing 50 mL of water through the machine at 5 mL/min.
5. Pass 50 mL of gel filtration buffer through the machine at 5 mL/min.
6. Connect the column to the machine and pass an additional 100 mL of gel filtration buffer.
7. Clean the injection valve by injecting 1 mL of gel filtration buffer.
8. Repeat Step B7 at least two more times.

C. Preparing the lipid-ANTS/DPX solution

1. Prepare a freezing solution in a beaker by adding dry ice to > 95% ethanol. The freezing solution is used to flash freeze the lipids containing ANTS/DPX. Alternatively, liquid nitrogen can be used to flash freeze. It is ideal to keep the freezing solution below -50°C.
2. Fill another beaker with lukewarm water for thawing the lipids.
3. Re-suspend lipids in 5 mM HEPES (pH 7.4), 1 mL for every 10 mg of lipids.
4. Vortex until the solution is well suspended.
5. Measure the solution volume and add 50 mM of ANTS and DPX into the tube.
6. Mix by pipetting up and down and cover the tube with aluminum foil.
7. Place the tube into the freezing solution and allow it to freeze.
8. Move the tube into warm water to thaw the lipids.
9. Repeat Steps C7-C8 for five more freeze/thaw cycles.

Note: Six freeze/thaw cycles are usually sufficient to solubilize the lipids. If problems occur during the extrusion, such as difficulties in passing the liquid through the membranes, perform more freeze/thaw cycles.

D. Producing liposomes via extrusion

1. Assemble the extruder according to the manufacturer's specifications.
2. Draw up 1 ml of gel filtration buffer using one of the syringes and pass the buffer through the extruder until the liquid has been transferred to the other syringe.

Note: You should feel a little bit of resistance. If there is no resistance, it is possible the membrane has

moved during assembly or has torn.

3. Remove the buffer from the syringe.
4. Draw up to 1 mL of liposomes into one of the syringes.
5. Pass the liposomes through the membrane until the liquid has been transferred from one syringe to the other.
6. Repeat Step D5 for a total of 20 times.
7. Inspect the fluid after extrusion; it should have changed from an opaque to a transparent, yellow-tinged liquid (Figure 1).

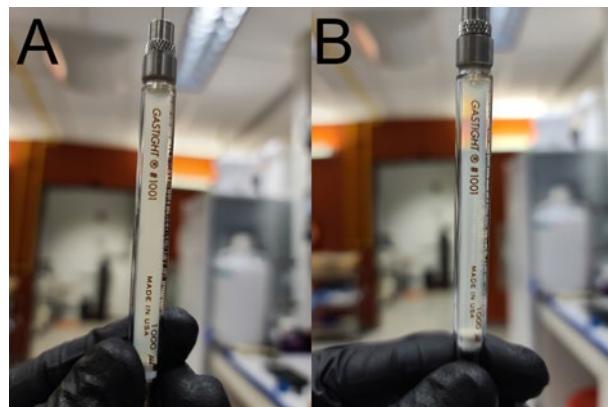


Figure 1. Comparison of the lipid solution before and after extrusion.

A. The lipid solution before extrusion has an opaque color. B. The lipid solution becomes more transparent after extrusion.

8. Dispense the lipids into a clean glass tube.

E. Removal of excess fluorescent dyes via desalting chromatography

1. After pre-equilibration of the column and machine and cleaning the injection loop, inject the liposomes into the injection loop.
2. Run the desalting program at a 0.5 mL/min flow rate, using gel filtration buffer, and collect 0.5 mL per fraction.
3. Allow 50 mL of buffer to pass through the column.
4. The liposomes will be eluted out of the column after approximately 2 mL have passed through.
5. Collect fractions containing the liposomes, usually in six tubes for a total 3 mL of liposomes (Figure 2).

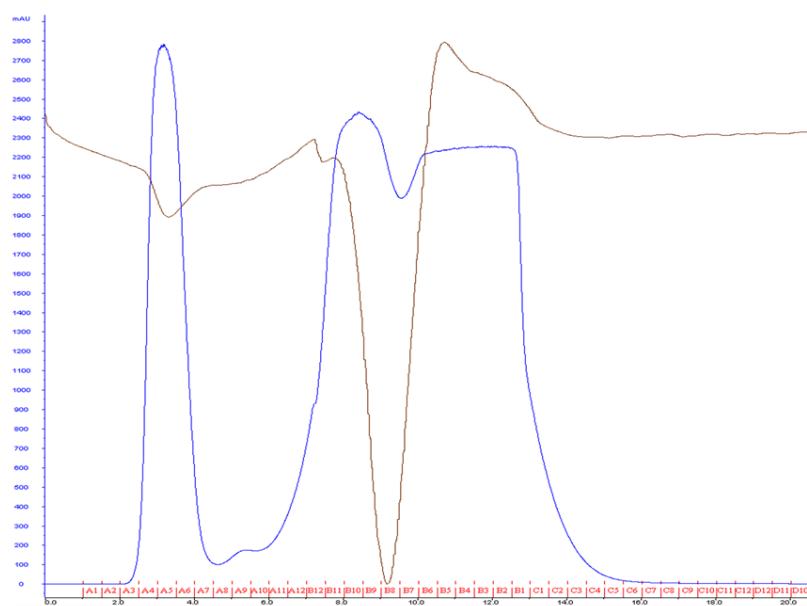


Figure 2. Most lipids will elute after flowing approximately 2 mL of buffer through the column.
A typical yield of 3 mL is expected using this procedure. The blue line represents mAu, and the brown line represents conductance.

6. Combine all liposome samples, cover with aluminum foil, and store at 4°C.
7. Liposomes at this stage can be stored for approximately 1-3 weeks.
8. Liposomes prepared with this procedure are usually consistent in concentration. However, technical replicates with the same batch of lipids are needed for every biological replicate to obtain consistent results.
9. Clean the column by passing at least 5 CV + 30 ml (the volume of the machine) of water through the machine.
10. Pass at least five CV in addition to 30 mL of 20% ethanol through the machine for column storage.

F. Liposome leakage assay

1. The quality of the liposomes should be tested before analyzing the protein of interest by adding 2% (v/v) Triton X-100, which completely lyses the liposomes, quickly revealing a strong fluorescence signal.
2. Set fluorometer excitation to 350 nm and record emissions at 520 nm and continue to measure at 1-second intervals for 5-10 min.
3. Cross polarizers are applied at the excitation and emission paths to reduce background. If necessary, apply a 515 nm long-pass filter at the emission path to reduce background scatter.
4. If available, connect a water bath to the water inlet and outlet of the sample chamber to maintain a constant temperature. This is especially important with proteins that interact at the physiological temperature, as is the case with EsxAB.
5. To a UV cuvette, add 1.15 mL of gel filtration buffer, 150 µL of 1 M NaAc buffer (pH 4), and 100 µL of liposomes.
6. Place the sample cuvette inside the sample chamber and close the lid.
7. After approximately 30 s, use a gel loading pipette tip to inject 100 µL of 2% Triton X-100 into the cuvette through the hole on the cover lid. The ANTS fluorescence should increase at least 5-fold (Figure 3). This will serve as a positive control.
 - a. The fluorescence intensity will drop for a second or two due to the dilution of the mix inside the cuvette, but it will begin to rise shortly after.

- b. A negative control consists of the sample prepared with liposomes and the gel filtration buffer.
8. Continue experimenting by testing cytolytic proteins; prepare the cuvette as in Step F5 (Figure 3).

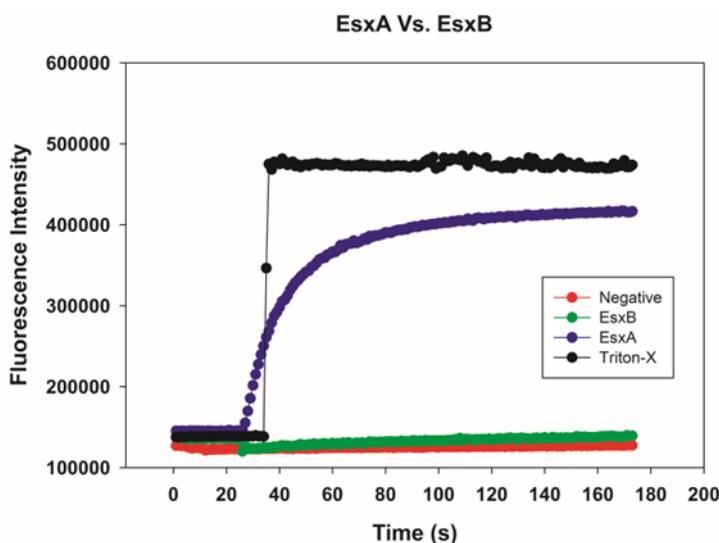


Figure 3. Raw plots of an ANTS/DPX assay.

The background fluorescence intensity is approximately 100,000 units. The fluorescence intensity increased 5-fold after adding 2% Triton X-100. With EsxA, a protein known to have MPA, we observed a gradual increase in fluorescence. EsxB, a protein without MPA, did not increase fluorescence intensity. The negative control consisting of the only gel filtration buffer did not increase fluorescence intensity.

9. Place the UV cuvette in the sample chamber and close the lid.
10. After about 30 s, use a gel loading pipette tip to add 100 μ L of protein into the cuvette.
- Protein concentrations can vary, but a total of 100 μ g is usually used in our experiments.
 - Depending on the nature of the experiment, the protein can be loaded into the cuvette instead of the pH 4 buffer. Then, after 30 s, adding the pH 4 buffer will cause EsxAB to lyse the membrane.

Data analysis

- The fluorescence intensity is normalized at the time point the protein was added by subtracting the lowest intensity value from all other values, removing the background fluorescence.
- The lowest intensity value is now deemed the measurement at $T = 0$, and all values from $T = 0$ until the end of the experiment are averaged for all replicates.
The dilution of the lipids by adding the sample results in a small drop in fluorescence. Measuring from the moment the protein is added allows determination of the increase in fluorescence as the liposomes are lysed.
- The average intensity is graphed against the time to obtain a curve (Figure 4A).
The plateau followed by a one-phase association can also be used to obtain an association constant.
- The average of the highest fluorescence intensity values for each sample are graphed in a bar graph (Figure 4B).

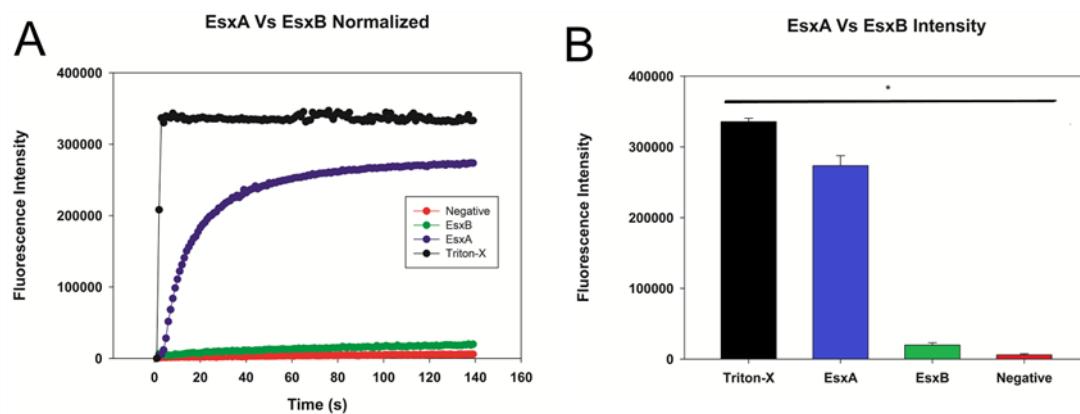


Figure 4. Plot of sample liposome experiment after normalization.

A. The data were normalized to remove background fluorescence and to account for the drop in fluorescence caused by the dilution of the liposomes by the sample. B. The peak fluorescence was graphed with error bars representing the standard deviation. Statistical significance is represented here as well.

5. The values are tested for normality using the Shapiro-Wilk test.
6. If the data fails to differ from normal, it is tested for significance with a One-way ANOVA; otherwise, a Tukey-Kramer test is performed.

Recipes

1. Gel filtration buffer

20 mM Tris, pH 7.4
10 mM NaCl

2. pH 4 Buffer

1 M NaAC, pH 4.01

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This protocol was adapted from Nieva *et al.*, 1989 (DOI: 10.1021/bi00444a032) and optimized for the EsxA/EsxB complex.

Competing interests

Authors have no competing interests to declare.

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Tracking the Subcellular Localization of Surface Proteins in *Staphylococcus aureus* by Immunofluorescence Microscopy

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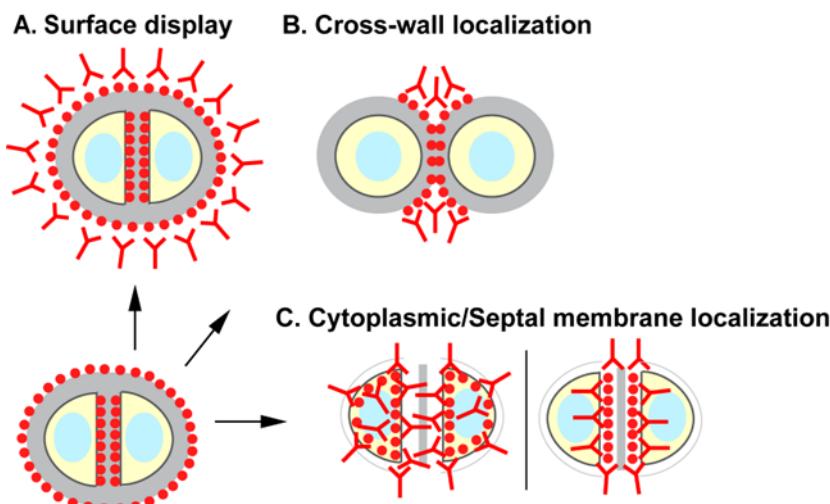
Abstract

Surface proteins of *Staphylococcus aureus* and other Gram-positive bacteria play essential roles in bacterial colonization and host-microbe interactions. Surface protein precursors containing a YSIRK/GXXS signal peptide are translocated across the septal membrane at mid-cell, anchored to the cell wall peptidoglycan at the cross-wall compartment, and presented on the new hemispheres of the daughter cells following cell division. After several generations of cell division, these surface proteins will eventually cover the entire cell surface. To understand how these proteins travel from the bacterial cytoplasm to the cell surface, we describe a series of immunofluorescence microscopy protocols designed to detect the stepwise subcellular localization of the surface protein precursors: surface display (protocol A), cross-wall localization (protocol B), and cytoplasmic/septal membrane localization (protocol C). Staphylococcal protein A (SpA) is the model protein used in this work. The protocols described here are readily adapted to study the localization of other surface proteins as well as other cytoplasmic or membrane proteins in *S. aureus* in general. Furthermore, the protocols can be modified and adapted for use in other Gram-positive bacteria.

Keywords: Immunofluorescence microscopy, *Staphylococcus aureus*, Surface proteins, YSIRK/GXXS signal peptide, Protein A (SpA), SecA, Surface display, Cross-wall localization, Septal localization

This protocol was validated in: eLife (2018); DOI: 10.7554/eLife.34092

Graphical Abstract:



Tracking the subcellular localization of surface proteins in *S. aureus*.

Background

Staphylococcus aureus is a Gram-positive bacterium and an opportunistic pathogen. It frequently colonizes human nares and skin and is a leading cause of both hospital- and community-acquired infections (von Eiff *et al.*, 2001; Tong *et al.*, 2015). The cell envelope of *S. aureus* consists of a cytoplasmic membrane and a thick cell wall peptidoglycan layer. To replicate, *S. aureus* undergoes binary fission by forming a division septum at the mid-cell. The cell wall biosynthesis machinery is recruited to the septum during cell division (Pinho and Errington, 2003). New cell wall peptidoglycan is synthesized to form a cross-wall ring and eventually a cross-wall disc coupled with the invagination of the septal membrane (Zhou *et al.*, 2015). Once the cross-wall disc is fully synthesized, specific cell wall hydrolases cleave at the outer edges of the cross-wall to split the two daughter cells (Oshida *et al.*, 1995; Sugai *et al.*, 1995; Yamada *et al.*, 1996; Kajimura *et al.*, 2005). Due to high internal turgor pressure, the two daughter cells separate from each other and the newly synthesized cross-wall discs become the new hemispheres of the daughter cells (Monteiro *et al.*, 2015; Zhou *et al.*, 2015).

Cell wall peptidoglycan-anchored surface proteins are key components of the Gram-positive bacterial cell envelope. Many of them perform virulence functions in *S. aureus*, such as adhesion, biofilm formation, nutrient acquisition, antibiotic resistance, and immune evasion (Foster *et al.*, 2014; Schneewind and Missiakas, 2019). Many surface protein precursors contain a specific N-terminal signal peptide with a highly conserved YSIRK/GXXS motif (Rosenstein and Götz, 2000; Tettelin *et al.*, 2001). The secretion, cell wall anchoring, and surface display of YSIRK/GXXS proteins are tightly coupled with the bacterial cell cycle (Carlsson *et al.*, 2006; Raz *et al.*, 2012; Yu *et al.*, 2018). In the early stages, the YSIRK/GXXS signal peptide promotes localized protein secretion at the division septum (Carlsson *et al.*, 2006; DeDent *et al.*, 2008). Subsequently, septal secreted surface proteins are covalently anchored to the cross-wall peptidoglycan by sortase A (Mazmanian *et al.*, 1999). Upon cell division and separation, cross-wall-anchored surface proteins are displayed on the surface of the new hemisphere of the daughter cells (Cole and Hahn, 1962; Swanson *et al.*, 1969; Raz *et al.*, 2012; Yu *et al.*, 2018). Eventually, surface proteins are displayed on the entire cell surface after several generations of cell division (DeDent *et al.*, 2008; Raz *et al.*, 2012; Yu *et al.*, 2018).

Proper imaging methods are essential in revealing the subcellular localization of proteins. Here, we describe a series of protocols to track the subcellular localization of surface proteins. While it is straightforward to localize proteins on bacterial cell surface (protocol A, Figure 1), a “pulse-chase” type of method is used to reveal the localization of newly anchored surface proteins. In their classical paper, Cole and Hahn (1962) described an

immunofluorescence staining method in which streptococcal cells were incubated with fluorescently labeled surface protein M antibody and subsequently with non-fluorescent antibody. In another classical study, streptococci were trypsin-treated to digest the existing M protein on the bacterial surface; new surface-deposited M protein was observed after re-incubating the bacteria in fresh medium without trypsin (Swanson *et al.*, 1969). The method of trypsinization followed by regeneration has subsequently been used to localize newly anchored surface proteins on the cell surface of both streptococci and staphylococci (Carlsson *et al.*, 2006; DeDent *et al.*, 2008; Raz *et al.*, 2012; Yu *et al.*, 2018). Here, we provide a detailed description of the protocol that is specifically tailored to *S. aureus* (protocol B, Figure 1). The model protein we use is protein A (SpA), one of the major staphylococcal surface proteins that binds to host immunoglobulin and disrupts host immune responses (Forsgren and Sjöquist, 1966).

We have previously shown that SpA engages the SecA-mediated secretion pathway for translocation across the cytoplasmic membrane (Yu *et al.*, 2018). To reveal where SpA precursors accumulate in the cytoplasm upon secA depletion, we developed a protocol to detect the localization of intracellular proteins (protocol C, Figure 1) based on methods described earlier by Harry *et al.*, (1995) and Pinho and Errington (2003). In this protocol, staphylococcal cells are fixed with paraformaldehyde and glutaraldehyde, which adhere to the poly-L-lysine-coated glass slide. Cells are digested on the slide with a robust staphylococcal cell wall hydrolase, lysostaphin, to generate protoplasts (Schindler and Schuhardt, 1964). The protoplasts are fixed and permeabilized with methanol and acetone, respectively, and subsequently subjected to immunofluorescence staining. Depending on the genetic background of different strains, protocol C can be used to localize membrane-bound or cytoplasmic-localized surface protein precursors. Furthermore, protocol C is not restricted to surface proteins; it can be used to localize cytoplasmic or membrane proteins in *S. aureus* in general. The protocols described here can also be adapted for use in other Gram-positive bacteria.

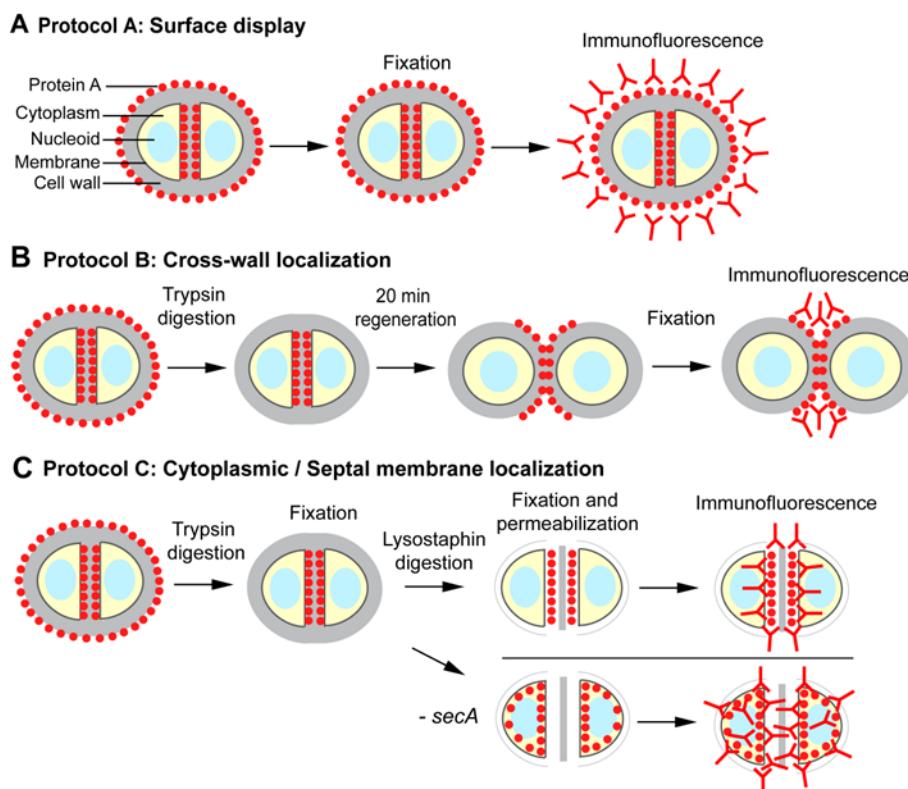


Figure 1. Schematic overview of the protocols described in this work

Materials and Reagents

1. 8-well glass slides (MP Biomedicals/Thermo Fisher Scientific, catalog number: 096040805E)
2. Disposable borosilicate glass tubes, 16 mm diameter, 125 mm length (Thermo Fisher Scientific, catalog number: 1496130)
3. 1,250 μ L XL graduated tips (USA Scientific, catalog number: 1112-1720)
4. 200 μ L graduated tips (USA Scientific, catalog number: 1110-1700)
5. 10 μ L graduated tips (USA Scientific, catalog number: 1111-3700)
6. 10 mL serological pipets (Thermo Fisher Scientific, catalog number: 1367811E)
7. 25 mL serological pipets (Thermo Fisher Scientific, catalog number: 13-678-11)
8. 1.5 mL microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-408-129)
9. 2.0 mL microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-408-138)
10. Scienceware microcentrifuge tube rack (Thermo Fisher Scientific, catalog number: 10029259)
11. 1.5 mL microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-408-129)
12. 2.0 mL microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-408-138)
13. Nunc 15 mL conical sterile tubes (Thermo Fisher Scientific, catalog number: 12565269)
14. Nunc 50 mL conical sterile tubes (Thermo Fisher Scientific, catalog number: 12565271)
15. Corning PES syringe filters (Thermo Fisher Scientific, catalog number: 09-754-29)
16. 20 mL filter syringes (Thermo Fisher Scientific, catalog number: 14-955-460)
17. Round Petri dishes (100 \times 15 mm) (Thermo Fisher Scientific, catalog number: FB0875712)
18. Transfer pipettes (Thermo Fisher Scientific, catalog number: 13-711-7M)
19. Kimberly-Clark Professional™ Kimwipes™ Delicate Task Wipers (Thermo Fisher Scientific, catalog number: 06-666A)
20. BD Bacto™ Tryptic Soy Broth (TSB) (Thermo Fisher Scientific, catalog number: DF0370-07-5)
21. BD Tryptic Soy Agar (TSA) (Thermo Fisher Scientific, catalog number: DF0369-07-8)
22. Sodium chloride (NaCl) (Thermo Fisher Scientific, catalog number: S271-1)
23. Hydrochloric acid (HCl) (Thermo Fisher Chemical, catalog number: 187066)
24. Potassium chloride (KCl) (Thermo Fisher Scientific, catalog number: AM9640G)
25. Potassium phosphate dibasic (K_2HPO_4) (Thermo Fisher Scientific, catalog number: BP363-500)
26. Sodium phosphate dibasic, anhydrous (Na_2HPO_4) (Thermo Fisher Scientific, catalog number: BP332-1)
27. Ethylenediamine tetraacetic acid, EDTA (Thermo Fisher Scientific, catalog number: BP118-500)
28. Tris base (Thermo Fisher Scientific, catalog number: BP152-5)
29. D-(+)-glucose (Sigma-Aldrich, catalog number: G8270-1KG)
30. Acetone (Thermo Fisher Scientific, catalog number: A929-4)
31. Methanol (Thermo Fisher Scientific, catalog number: A454-4)
32. Ethanol (Thermo Fisher Scientific, catalog number: A405P-4)
33. Bovine Serum Albumin (BSA) (Thermo Fisher Scientific Bioreagents, catalog number: BP1600-100)
34. 0.1% poly-L-lysine solution (Sigma-Aldrich, catalog number: P8920-100ML)
35. Paraformaldehyde (PFA) 4% in PBS (Thermo Fisher Scientific, catalog number: AAJ19943K2)
36. Glutaraldehyde 50% in H₂O (Sigma-Aldrich, catalog number: 340855-25ML)
37. Primary antibody: SpAKKAA antiserum (Kim *et al.*, 2010). Store at 4°C
38. Secondary antibodies:
 - a. Goat anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, catalog number: A-11034). Store at 4°C
 - b. Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Thermo Fisher Scientific, catalog number: A-21244). Store at 4°C
39. Molecular Probes™ SlowFade™ Diamond Antifade Mountant (Invitrogen, catalog number: S36963)
40. Nile Red (Sigma-Aldrich, catalog number: 19123-10MG)
41. Hoechst 33342 DNA dye, 10 mg/ml (Thermo Fisher Scientific, catalog number: H3570), store at 4°C.
42. BODIPY™ Vancomycin-FL (Thermo Fisher Scientific, catalog number: V34850)
43. Corning™ Rectangular Cover Glasses No.1 (22 \times 50 mm) (Thermo Fisher Scientific, catalog number: 12-553-461) (see Note 1)

44. Clear nail polish (cheapest one in any grocery store)
45. Trypsin, from bovine pancreas (Sigma-Aldrich, catalog number: T1426)
46. Trypsin inhibitor, from glycine max soybean (Sigma-Aldrich, catalog number: T9128)
47. Lysostaphin (AMBI, catalog number: LSPN-50)
48. Phosphate-buffered saline (PBS) (see Recipes)
49. Fixation solution (see Recipes)
50. GTE solution (see Recipes)
51. Trypsin stock solution (see Recipes)
52. Trypsin inhibitor stock solution (see Recipes)
53. BSA blocking solution (see Recipes)
54. Lysostaphin stock solution (see Recipes)
55. Nile Red stock solution (see Recipes)
56. BODIPYTM Vancomycin-FL stock solution (see Recipes)

Equipment

1. Eppendorf pipettes 100–1,000 µL, 20–200 µL, 2–20 µL, 0.1–2.5 µL (Eppendorf, catalog number: 2231000714)
2. Eppendorf Easypet[®]3 (Eppendorf, catalog number: 4430000018)
3. FisherbrandTM TraceableTM Multi-Colored Timer (Thermo Fisher Scientific, catalog number: 02-261-840)
4. Forceps (MilliporeSigmaTM Filter Forceps/Thermo Fisher Scientific, catalog number: XX6200006P)
5. In-house vacuum
6. Shaker (Eppendorf New BrunswickTM Innova[®] 42 shaker, catalog number: EPM1335-0010)
7. Table centrifuge (Eppendorf, model: Centrifuge 5425, catalog number EP5405000441)
8. Spectrophotometer (Thermo Scientific Genesys GENESYSTM 30 Visible Light Spectrophotometer, catalog number: 14-380-442)
9. Mini-tube rotator (FisherbrandTM Mini Tube Rotator, catalog number: 88-861-051)
10. Incubator microbiological (Fisher Scientific, catalog number: 51030513)
11. -20°C freezer
12. 4°C refrigerator
13. LP Vortex Mixer (Thermo Fisher Scientific, catalog number: 88880017)
14. Leica SP5 2-photon Laser Scanning Confocal microscope (Leica Microsystems, product name: Leica TCS SP5 Confocal)

Software

1. Image J (Rasband W.S./U. S. NIH, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>)
2. Leica microscope software LAS_AF Leica (Leica microscopes)
3. Prism GraphPad Software for statistical analysis (<https://www.graphpad.com/scientific-software/prism/>)
4. Adobe Illustrator to assemble figures

Procedure

A. Slide preparation

1. Add 50 µL 0.1% poly-L-lysine to each well of an 8-well glass slide and allow to sit for 5 min at room temperature (see Note 2).
2. Briefly rinse the wells with ddH₂O, remove the excess liquid using a vacuum, and allow the slide to air

dry completely (see Notes 3 and 4).

B. Bacterial cultures

1. Prepare an overnight culture: inoculate one single colony from a streaked agar plate to 3 mL TSB in a glass test tube; add the appropriate antibiotics, if needed.
2. Grow the overnight culture at 37°C with rigorous shaking at 220 rpm.
3. The next morning, inoculate 30 µL overnight culture to 3 mL fresh TSB (1:100 dilution).
4. Grow the cultures at 37°C with rigorous shaking at 220 rpm.
5. Measure the optical density OD₆₀₀ of the culture every hour in a spectrophotometer.
6. Place the cultures on ice when OD₆₀₀ reaches 0.8–1.0 (see Note 5).
7. Continue the sample preparation in Part C below according to the different protocols (A, B, or C).

C. Sample preparation

Protocol A – Surface display:

1. Transfer 2 ml bacterial culture into a 2 ml microcentrifugation tube.
2. Spin at 18,000 × g for 3 min in a tabletop centrifuge to pellet the bacterial cells.
3. Remove the supernatant without disturbing the pellet.
4. Resuspend the pellet in 1 mL PBS and vortex well.
5. Spin at 18,000 × g for 3 min and remove the supernatant (steps 4–5 are “wash with PBS” steps).
6. Resuspend the pellet in 1 mL PBS, vortex thoroughly; mix 250 µL bacterial suspension with 250 µL fixation solution (see Recipes) in a clean 1.5 mL microcentrifuge tube, briefly vortex to mix, and incubate for 20 min at room temperature (fixation step).
7. Wash twice with PBS, as described in steps 4–5 (see Note 6).
8. Resuspend the pellet in 150 µL PBS, vortex thoroughly (see Note 7).
9. Add 50 µL bacterial suspension to poly-L-lysine-coated glass slides and allow to sit for 5 min.
10. Remove the excess liquid (non-adherent cells) using a vacuum.
11. Add one drop of PBS to each well using a plastic disposable transfer pipette and remove using a vacuum (this is the “drop and remove on-slide wash” step).
12. Repeat the drop and remove on-slide wash step.
13. Continue with immunofluorescence in Part D.

Protocol B – Cross-wall localization:

1. Transfer 2 mL bacterial culture into a 2 mL microcentrifugation tube.
2. Spin at 18,000 × g for 3 min in a tabletop centrifuge to pellet the bacterial cells.
3. Remove the supernatant without disturbing the pellet.
4. Wash once with PBS.
5. Resuspend the pellet in 900 µL PBS, vortex thoroughly; add 100 µL 5 mg/mL trypsin stock solution (see Recipes) and briefly vortex (trypsin final concentration: 0.5 mg/ml).
6. Incubate the tubes in a Mini-tube rotator at 37°C for 1 h at a rotation speed of 16 (medium speed).
7. Wash twice with PBS.
8. Resuspend the pellet in 900 µL fresh TSB, vortex thoroughly; add 100 µL 10 mg/mL soybean trypsin inhibitor stock solution (see Recipes) and briefly vortex to mix (final concentration of soybean trypsin inhibitor: 1 mg/mL).
9. Incubate the tubes in a Mini-tube rotator at 37°C for exactly 20 min at a rotation speed of 16 (see Note 8).
10. Add 250 µL fixation solution to a clean 1.5 mL microcentrifuge tube during the 20 min incubation.
11. At the 20-min timepoint, quickly transfer 250 µL bacterial sample to the microcentrifuge tubes prepared in the previous step.
12. Vortex to mix and allow the sample to sit at room temperature for 20 min.

13. Wash twice with PBS.
14. Resuspend the pellet in 150 µL PBS and vortex thoroughly (adjust the volume depending on the pellet size).
15. Add 50 µL bacterial suspension to a glass slide coated with poly-L-lysine and allow to sit for 5 min.
16. Remove the liquid (non-adherent cells) using a vacuum.
17. Perform the drop and remove on-slide wash twice for each well as described above.
18. Continue with immunofluorescence in Part D.

Protocol C – Cytoplasmic/septal membrane localization:

1. Place two 50 mL tubes containing approximately 25 mL methanol and 25 mL acetone, respectively, into a -20°C freezer.
2. Normalize all the bacterial cultures to OD₆₀₀ = 1.
3. Transfer 2 mL normalized bacterial culture to a 2 mL microcentrifuge tube (this step is to have same cell numbers for the following enzymatic digestion step).
4. Spin at 18,000 × g for 3 min in a tabletop centrifuge to pellet the bacterial cells.
5. Remove the supernatant without disturbing the pellet.
6. Wash once with PBS.
7. Resuspend the pellet in 900 µL PBS and vortex thoroughly; add 100 µL 5 mg/mL trypsin stock solution and briefly vortex (see Note 9).
8. Incubate in a Mini-tube rotator at 37°C for 1 h at a rotation speed of 16.
9. Wash twice with PBS.
10. Resuspend the pellet in 500 µL PBS, vortex thoroughly; add 500 µL fixation solution and vortex to mix.
11. Incubate the sample for 15 min at room temperature and then on ice for 15 min to fix.
12. Wash three times with PBS.
13. Resuspend the pellet in 1 ml freshly made GTE buffer (see Recipes) and vortex thoroughly (see Note 10).
14. Add 50 µL cell suspension to poly-L-lysine-coated glass slides (this is the control without lysostaphin digestion) (see Note 11).
15. Prepare a timer, add 10 µL lysostaphin working solution (see Recipes) to the rest of the cell suspension; quickly vortex and immediately add 50 µL to the glass slides (see Note 12).
16. Incubate for 2 min on the slide (see Note 13).
17. Remove the liquid using a vacuum until completely dry.
18. Immediately place the slide into prechilled methanol at -20°C for 5 min.
19. Take out the slide using forceps and place into prechilled acetone at -20°C for 30 s (see Note 14).
20. Take out the slide using forceps and allow to air-dry completely.
21. Once the slide is dry, apply 50 µL PBS to the sample well to rehydrate.
22. Perform the drop and remove on-slide wash twice for each well.
23. Continue with immunofluorescence in Part D.

D. Immunofluorescence

1. Remove PBS, add BSA blocking solution (see Recipes), and incubate for 30 min at room temperature.
2. Remove the blocking solution, add 50 µL primary antibody solution (rabbit serum SpAKKAA 1:4,000 dilution in BSA blocking solution), and incubate overnight at 4°C or at room temperature for 1 h (see Note 15).
3. Remove the unbound primary antibody solution and wash 8 times with PBS with the last wash step for 5 min.
4. Remove the washing solution, add 50 µL secondary antibody diluted in BSA blocking solution (e.g., Alexa Fluor 647-IgG or Alexa Fluor 488-IgG, 1:500 dilution), and incubate in the dark for 1 hour at room temperature (see Note 16).
5. Perform the on-slide wash 10 times with PBS.
6. Take a clean 1.5 mL microcentrifuge tube, add 1 mL PBS, 5 µL Hoechest stock solution (1:200 dilution),

- 2 μ L Van-FL stock solution (1:500 dilution), or 5 μ L Nile Red stock solution (1:200 dilution) (see Recipes) and mix well; add 50 μ L staining solution to each well.
7. Incubate in the dark for 10 min at room temperature.
 8. Perform the on-slide wash three times with PBS.
 9. After the last wash, remove all the excess liquid from the well.
 10. Add a 5- μ L drop of Slow Fade Diamond Antifade Mountant at 3 different places between the sample wells (see Note 17).
 11. Brush a thin layer of nail polish around the edges of the slide and seal with a cover slip; gently press the cover slip and use a Kim wipes to remove the excess antifade solution around the edges (see Note 18).
 12. Image the samples using a microscope with the appropriate fluorescent channels (Part E).
 13. The prepared slides can be stored at 4°C for a few days and at -20°C for a longer period; however, immediate imaging is recommended.

E. Imaging

1. The samples prepared above are suitable to be imaged by different imaging systems, including epifluorescence microscopy, confocal microscopy, or deconvolution microscopy. However, to reveal bacterial cellular features and define protein localization in tiny bacterial cells, a microscope with high resolution is recommended. A 60 \times or 100 \times objective lens with a higher numerical aperture is needed. We used a Leica DM 2000 coupled with a sensitive CCD camera, a Leica SP5 2-photon Laser Scanning Confocal microscope, and a Leica SP8 3X STED Laser Confocal Microscope. All showed good imaging results.
2. Representative images of protocols A, B, and C are displayed in Figure 2. The images were captured using a Leica SP5 2-photon Laser Scanning Confocal microscope.

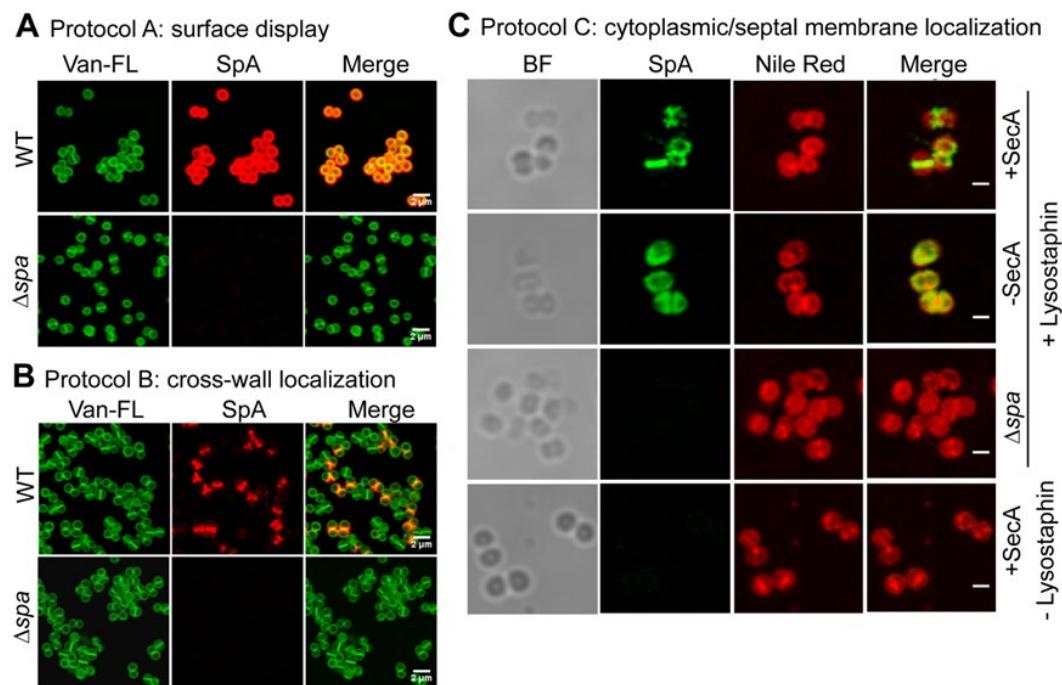


Figure 2. Representative images from (A) protocol A, showing surface display of SpA; (B) protocol B, showing cross-wall localization of SpA; and (C) protocol C, showing septal localization of SpA in the presence of SecA and cytoplasmic localization of SpA precursors in the absence of SecA.
 Van-FL: BODIPY™ Vancomycin-FL cell wall staining; BF: brightfield images; Nile Red: membrane staining; scale bar: 2 μ m in panels A and B and 1 μ m in panel C. Images are adapted from Yu *et al.* (2018).

Data analysis

1. Take at least three images with random views for each sample in each experiment. One has to take more random images, especially when there are only a few cells on the slides or when there are different phenotypes on one slide.
 2. Perform the experiment independently at least three times.
 3. To quantitate the percentage of SpA cross-wall localization, open images from protocol B in ImageJ, split the channels, and enlarge the images to allow better visualization.
 4. Open the “cell counter” tool in ImageJ.
Select cell type 1, manually count pairs of diplococci in Van-FL-stained images, and record the number. Count at least 50 pairs of diplococci per image. Diplococci are defined as two connecting daughter cells that have just been split but not yet separated (see sample images in Figure 3) (see Note 19).
 5. Select cell type 2, manually count cross-wall localized SpA signals in the merged images, and record the number. Cross-wall localized SpA signals are defined as clear lines at the cross-wall. To be rigorous, dots are not counted.
 6. Calculate the average of three images per experiment.
 7. Input the average values of three independent experiments to GraphPad Prism.
 8. In GraphPad Prism, use a *t*-test to statistically analyze significant differences between two groups; use one-way ANOVA for multiple group comparisons; and use Tukey’s multiple comparison test to analyze differences among multiple groups.

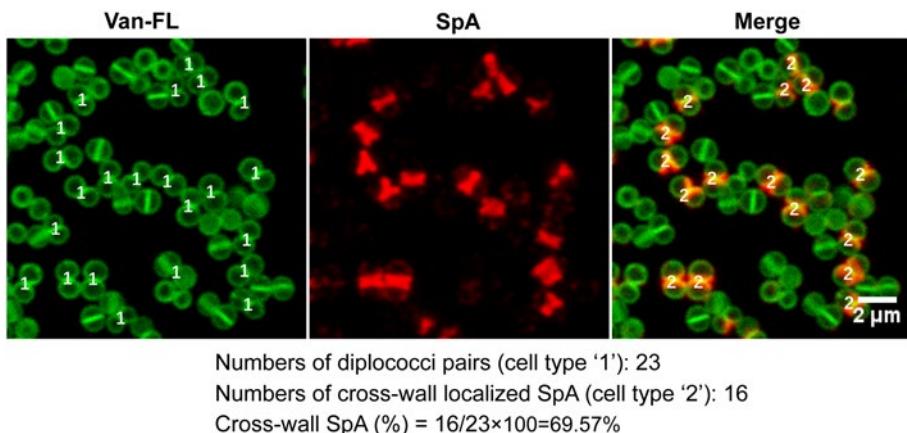


Figure 3. Sample images with the cell counting window, demonstrating how to quantitate the percentage of SpA cross-wall localization.

Images are adapted from Yu *et al.* (2018).

Notes

1. The choice of thickness of the cover slip depends on the imaging system.
 2. A 1:10 dilution of poly-L-lysine to 0.01% also works.
 3. It takes about 15 min to air-dry the poly-L-lysine-coated slides. One can also use a vacuum to dry the slides.
 4. To assemble the vacuum system, connect an in-house vacuum to a tube, cut the extremity off a 200 μ l pipette tip, and insert the tip into the tube. It is important not to touch the samples on the glass slides during drying.
 5. It usually takes about 2–3 h for *S. aureus* to reach an OD₆₀₀ of 0.8–1.0 under standard lab culture conditions.
 6. Cells tend to clump after fixation; a longer vortex may be needed.
 7. The volume can be adjusted according to the pellet size; if unsure, one can add less PBS and make dilutions. The key point here is to have a proper number of cells on the slide so that most of the cells are well separated. Too many cells will lead to bacterial clumping and cause artifacts in immunostaining; too few cells will not

- provide reliable results.
8. The time of re-generation was determined experimentally in our protocol. It should be tested and optimized depending on different growth conditions and antigens.
 9. This step is not critical for protocol C, as lysostaphin digestion will remove most of the cell wall as well as the existing SpA. We include this step in our protocol to minimize any potential background caused by existing SpA.
 10. GTE buffer is an osmotic stabilizing buffer. Lysostaphin is a zinc-dependent endopeptidase (Sabala *et al.*, 2014). Although EDTA in the GTE buffer can chelate zinc, it does not have any obvious negative effects in our experiments. We have tried other osmotic stabilizing buffers without EDTA, such as TSM [50 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 10 mM MgCl₂], which also works.
 11. It is important to have this control. Staphylococcal cells after lysostaphin digestion will become more translucent in brightfield images, whereas undigested cells have a dark cell contour. Moreover, the two closely attached daughter cells will separate after lysostaphin digestion (see Figure 2C).
 12. Other cell wall hydrolases can substitute lysostaphin if this protocol is to be adapted for another bacterium. Lysozyme, for example, has been used in *Bacillus subtilis* (Harry *et al.*, 1995). Most *S. aureus* strains are lysozyme-resistant, which limits its use in *S. aureus*. The digestion time and buffer will have to be adjusted experimentally for a different enzyme or bacterium.
 13. It is critical to perform on-slide digestion to stabilize the protoplasts.
 14. This step stabilizes the protoplast after lysostaphin digestion and permeabilizes the cytoplasmic membrane. Depending on the bacterial strain and abundance of antigens, Triton X-100 can be used to further permeabilize the cytoplasmic membrane.
 15. For any new antigen, serial dilution of primary antibody is necessary to determine the optimal concentration. A negative control that does not express the antigen is an essential control. If there is no mutant available, one should include at least a control without primary antibody. Minimal background signals should be seen in the negative control.
 16. Depending on the microscope system, one can choose different fluorescent-labeled secondary antibodies. We consistently use the secondary antibody at a 1:500 dilution.
 17. Different kinds of antifade solution are commercially available. One should choose the antifade solution compatible with the imaging system.
 18. Make sure that the antifade solution covers every well as a very thin layer without bubbles, and that the cover slip is leveled on the slide.
 19. The reason for counting diplococci is because cross-wall localized SpA signals can only be detected at the cross-wall of these diplococci under our experimental conditions; however, as defining diplococci may be subjective, it can introduce bias. Thus, one can count total cell numbers instead of diplococci.

Recipes

1. Phosphate-buffered saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

1.8 mM K₂HPO₄

pH 7.4

a. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g K₂HPO₄ in 1 L ddH₂O

b. Adjust the pH to 7.4 with HCl and autoclave at 121°C for 20 min

2. Fixation solution

2.4% paraformaldehyde and 0.01% glutaraldehyde in PBS

Mix 30 mL 4% PFA and 10 μL 50% glutaraldehyde and add PBS to a 50-mL total volume. Store at 4°C (stable for at least two weeks).

3. GTE solution

50 mM glucose
10 mM EDTA
20 mM Tris-HCl pH 7.5

Note: Make fresh and filter-sterilize before use.

- a. Make stock solutions of 0.5 M EDTA (pH 8) and 1 M Tris-HCl (pH 7.5)
- b. Add 0.9 g D-glucose, 2 mL 0.5 M EDTA, and 2 mL 1 M Tris-HCl to a final volume of 80 mL ddH₂O
- c. Adjust the pH to 7.5 with HCl, add ddH₂O to 100 mL, filter-sterilize, and store at 4°C

4. T Trypsin inhibitor stock solution

5 mg/mL trypsin in PBS, filter-sterilize, and store at -20°C

5. Trypsin stock solution

10 mg/mL trypsin inhibitor in ddH₂O, filter-sterilize, store at -20°C

6. BSA blocking solution: 3% BSA in PBS

Dissolve 0.3 g BSA powder in 10 mL PBS; make fresh and filter-sterilize before use, store at 4°C

7. Lysostaphin stock solution

- a. Make a stock solution of 10 mg/mL in 20 mM sodium acetate (pH 4.5), store at -20°C
- b. Dilute with 200 mM Tris-HCl (pH 8) to 2 mg/mL as a working solution, store at 4°C

8. Nile Red stock solution

- a. Dissolve in 100% ethanol to make a 0.5 mg/mL stock solution, store at -20°C
- b. Add 5 µL Nile Red stock solution to 1 mL PBS (1:200 dilution) to stain the samples

9. BODIPY™ Vancomycin-FL stock solution

- a. Dissolve 100 µg in 100 µl DMSO to make a 1 µg/µL stock solution, store at -20°C
- b. Add 2 µL Van-FL stock solution to 1 mL PBS (1:500 dilution) to stain the samples

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Competing interests

The authors declare that there are no conflicts of interest or competing interests.

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Visualization of Host Cell Kinase Activation by Viral Proteins Using GFP Fluorescence Complementation and Immunofluorescence Microscopy

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Abstract

Non-receptor protein-tyrosine kinases regulate cellular responses to many external signals and are important drug discovery targets for cancer and infectious diseases. While many assays exist for the assessment of kinase activity *in vitro*, methods that report changes in tyrosine kinase activity in single cells have the potential to provide information about kinase responses at the cell population level. In this protocol, we combined bimolecular fluorescence complementation (BiFC), an established method for the assessment of protein-protein interactions, and immunofluorescence staining with phosphospecific antibodies to characterize changes in host cell tyrosine kinase activity in the presence of an HIV-1 virulence factor, Nef. Specifically, two Tec family kinases (Itk and Btk) as well as Nef were fused to complementary, non-fluorescent fragments of the Venus variant of YFP. Each kinase was expressed in 293T cells in the presence or absence of Nef and immunostained for protein expression and activity with anti-phosphotyrosine (pTyr) antibodies. Multi-color confocal microscopy revealed the interaction of Nef with each kinase (BiFC), kinase activity, and kinase protein expression. Strong BiFC signals were observed when Nef was co-expressed with both Itk and Btk, indicative of interaction, and a strong anti-pTyr immunoreactivity was also seen. The BiFC, pTyr, and kinase expression signals co-localized to the plasma membrane, consistent with Nef-mediated kinase activation in this subcellular compartment. Image analysis allowed calculation of pTyr-to-kinase protein ratios, which showed a range of responses in individual cells across the population that shifted upward in the presence of Nef and back down in the presence of a kinase inhibitor. This method has the potential to reveal changes in steady-state non-receptor tyrosine kinase activity and subcellular localization in a cell population in response to other protein-kinase interactions, information that is not attainable from immunoblotting or other *in vitro* methods.

Keywords: Protein-tyrosine kinase, Tec family kinases, Interleukin-2-inducible T cell kinase (Itk), Bruton's tyrosine kinase (Btk), HIV-1 Nef, Bimolecular fluorescence complementation (BiFC), Confocal microscopy, Signal transduction, Protein-protein interaction

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Background

Non-receptor protein-tyrosine kinases, exemplified by members of the Src and Tec kinase families, regulate many aspects of cell biology including growth, differentiation, and motility in response to diverse stimuli (Amatya *et al.*, 2019). Methods that assess the spatial and temporal aspects of tyrosine kinase signaling at the single cell and population levels are essential to better understanding their function. In this protocol, we provide details of a cell-based method to evaluate protein-protein interaction, kinase activity, and subcellular localization of Tec family kinases in response to the interaction with the HIV-1 accessory protein, Nef. This approach is potentially applicable to many other kinase systems in which protein-protein interactions impact kinase activity.

Nef is a small (27-34 kDa, depending on the subtype) membrane-associated protein unique to the primate lentiviruses HIV-1, HIV-2, and SIV (Foster and Garcia, 2008). HIV-1 Nef enhances viral infectivity, supports high-titer replication *in vivo*, and promotes immune escape of HIV-infected cells (Basmaciogullari and Pizzato, 2014; Pawlak and Dikeakos, 2015). Rhesus macaques infected with nef-defective SIV exhibit very low viral loads and do not progress to simian AIDS (Kestler *et al.*, 1991), illustrating an essential role for Nef in viral pathogenesis. Along the same lines, individuals infected with nef-defective HIV-1 can remain AIDS-free in the absence of antiretroviral therapy for many years (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995).

Nef lacks intrinsic biochemical activities, functioning instead through interactions with host cell proteins related primarily to endocytic trafficking and kinase signaling pathways (Staudt *et al.*, 2020). Nef hijacks non-receptor tyrosine kinases of the Src and Tec families normally linked to immune receptor activation to enhance HIV-1 replication. Nef directly activates the Src family members Hck and Lyn by binding to their SH3 domains (Briggs *et al.*, 1997; Trible *et al.*, 2006). Selective inhibition of Nef-mediated Src family kinase activation blocks Nef-dependent enhancement of HIV-1 infectivity and replication (Emert-Sedlak *et al.*, 2009 and 2013). Tec family kinases play essential roles in B- and T-cell receptor signaling (Andreotti *et al.*, 2010), with the interleukin-2 inducible T-cell kinase (Itk) and Bruton's tyrosine kinase (Btk) expressed in primary HIV-1 target cells (CD4⁺ T cells and macrophages, respectively). Readinger *et al.* provided the first evidence linking Itk to HIV-1 entry, viral transcription, assembly, and release (Readinger *et al.*, 2008). A subsequent study showed that Nef provides a link between HIV-1 infection and Tec family kinase signaling by demonstrating direct interaction between Nef and both Itk and Btk at the cell membrane (Taraifdar *et al.*, 2014). Treatment of HIV-infected T cells with a selective Itk inhibitor blocked Nef-dependent enhancement of viral infectivity and replication. Importantly, activation of both Src and Tec family kinases is highly conserved across all M group HIV-1 subtypes, consistent with an important function in the HIV-1 life cycle and viral pathogenesis (Narute and Smithgall, 2012; Emert-Sedlak *et al.*, 2013; Taraifdar *et al.*, 2014). For an in-depth review of Nef interactions with host cell tyrosine kinases, see Staudt *et al.* (2020).

In a recent study, we explored the molecular mechanisms of Tec family kinase activation by the Nef proteins of HIV-1 and SIV (Li *et al.*, 2020). By combining cell-based bimolecular fluorescence complementation (BiFC) (Romei and Boxer, 2019) and anti-phosphotyrosine immunofluorescence microscopy, we found that HIV-1 Nef interacts with Itk and Btk at the cell membrane and results in constitutive kinase activity. For the BiFC assay, Itk or Btk and Nef were fused to complementary, non-fluorescent fragments of the Venus variant (Nagai *et al.*, 2002) of YFP. The kinases were then expressed in 293T cells either alone or together with Nef, followed by immunostaining for Nef and kinase protein expression as well as protein-tyrosine phosphorylation using anti-phosphotyrosine (pTyr) antibodies. Multi-color confocal microscopy enabled simultaneous assessment of Nef-kinase complex formation (BiFC), kinase activity (anti-pTyr immunofluorescence), and kinase protein expression (anti-kinase immunofluorescence). When expressed alone, both Itk and Btk showed a diffuse subcellular staining pattern with the anti-kinase antibody and weak reactivity with the anti-pTyr antibody. In contrast, co-expression with Nef induced a strong BiFC signal with both Itk and Btk, indicative of interaction, and a strong anti-pTyr immunoreactivity was seen. The BiFC, pTyr, and kinase expression signals co-localized to the cell membrane, consistent with Nef-mediated kinase activation in this subcellular compartment. As a control, cells were treated with Tec family kinase inhibitors (Lin *et al.*, 2004; Roskoski, 2016) that suppressed the pTyr signal but did not affect BiFC, demonstrating that interaction of Nef with Tec family kinases at the membrane does not require kinase activity.

Results were quantitated at the single-cell level using the NIH ImageJ image analysis software (Schneider *et al.*, 2012). Kinase expression and tyrosine phosphorylation immunofluorescence signal intensities for at least 100 cells for each condition were expressed as the mean pTyr-to-kinase protein fluorescence intensity ratios. This analysis

enabled statistical comparisons of cell populations and showed that cells co-expressing Itk or Btk and Nef had significantly higher fluorescence ratios as compared with those expressing the kinase alone or the inhibitor-treated cells expressing the Nef-kinase complex.

Using the same approach, we also investigated Nef-stimulated Itk and Btk autophosphorylation on their respective activation loop tyrosine residues (pTyr511 and pTyr551, respectively), a step required for Tec family kinase activation (Joseph *et al.*, 2013). Cells were transfected with the Nef and Itk/Btk Venus fusion constructs for BiFC as before, but in this case the cells were stained with phosphospecific antibodies for the activation loop phosphotyrosines in place of the general anti-pTyr antibody. Co-expression with Nef led to significant increases in Itk and Btk activation loop autophosphorylation, which also localized almost exclusively to the cell membrane. SIV Nef (mac239 allele) was also found to strongly induce membrane-associated autophosphorylation of both kinases, demonstrating that Tec family kinase activation is conserved across Nef proteins from diverse primate lentiviruses. A representative confocal image from this study is shown in Figure 1.

While the experimental approach described above was developed to explore kinase interaction with, and activation by, a viral protein in a cell-based setting, the overall concept should be readily adaptable to any combination of kinases and interacting partners. The cell-based approach has important advantages over older *in vitro* methods such as immune-complex kinase assays, which do not provide information about the subcellular localization of the active kinase complex or the range of responses across a population of individual cells. However, one important caveat includes consideration of where to add the fragments of Venus for the BiFC assay. For example, both Nef and Tec family kinases localize to the cell membrane by virtue of N-terminal signals. Nef is myristoylated on its N-terminus, while Tec kinases have an N-terminal Pleckstrin homology (PH) domain that binds to membrane phosphoinositides. To avoid interference with these membrane-targeting signals, we were careful to fuse the Venus fragments to the C-terminus of each protein. Control experiments are also essential to verify that Venus fragment fusion does not influence basal kinase activity or localization, which is readily accomplished by comparing unfused with fused versions of each kinase in transfected cells and staining with kinase and phosphospecific antibodies. Finally, it should be noted that the Venus fluorophore, once reconstituted via BiFC, is irreversible. While this feature of BiFC may help to stabilize transient interactions for endpoint assessment by microscopy as described here, other techniques are more appropriate to assess the kinetics of interaction, such as the split-FAST reversible complementation system (Tebo and Gautier, 2019).

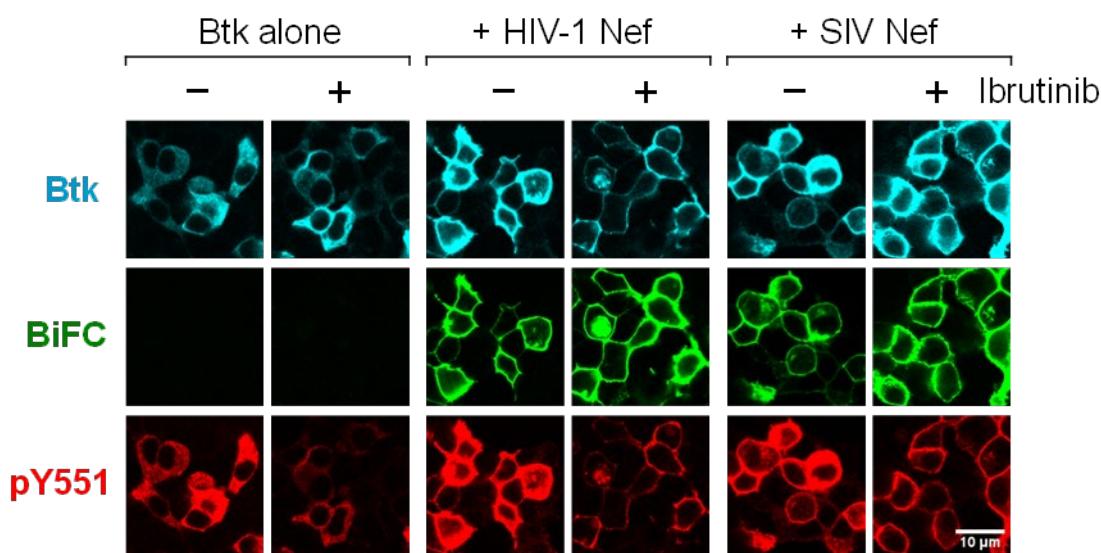


Figure 1. HIV-1 and SIV Nef proteins induce constitutive Btk activation loop autophosphorylation at the cell membrane.

Btk was expressed in 293T cells either alone or together with HIV-1 Nef (SF2 isolate) or SIV Nef (mac239) as BiFC pairs in the absence or presence of the Btk/Itk inhibitor ibrutinib (1 μM). Cells were fixed and stained for confocal microscopy with phosphospecific antibodies against the Btk activation loop phosphotyrosine (pY551;

red) and the Btk protein (V5 epitope; cyan). Nef interaction with Btk is observed as fluorescence complementation of the YFP variant, Venus (BiFC; green). Note that interaction and kinase activation occur at the plasma membrane.

Materials and Reagents

1. Phusion high-fidelity DNA polymerase (New England Biolabs, catalog number: M0530S)
2. Venus template (gift from Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan)
3. HIV-1 (SF2 allele) and SIV (mac239) Nef clones (NIH AIDS Reagent Program, HIV #11431; SIV #2476)
4. Full-length human Tec family kinase cDNA clones (Dana-Farber/Harvard Cancer Center PlasmID DNA Resource Core, Btk # HsCD00346954; Itk # HsCD00021352)
5. Mammalian expression vector, pCDNA3.1(−) (Thermo Fisher, catalog number: V79520)
6. Anti-V5 tag mouse monoclonal antibody (Thermo Fisher, catalog number: R960-25)
7. Anti-V5 tag rabbit polyclonal antibody (Sigma, catalog number: AB3792)
8. BTK anti-pY551 rabbit monoclonal antibody (Abcam, catalog number: ab40770)
9. Anti-pTyr antibody pY99 (Santa Cruz, catalog number: sc-7020)
10. Anti-HIV-1 Nef monoclonal antibody 6.2 (NIH AIDS Reagent Program, catalog number: 1539)
11. Goat anti-rabbit IgG (H+L), mouse/human ads-TXRD (Texas Red conjugate; cross-adsorbed to mouse and human immunoglobulins; Southern Biotech, catalog number: 4050-07)
12. Goat anti-mouse IgG (H+L), human ads-TXRD (Texas Red conjugate; cross-adsorbed to human immunoglobulins; Southern Biotech, catalog number: 1031-07)
13. Pacific Blue goat anti-mouse IgG antibody (Thermo Fisher/Molecular Probes, catalog number: P31582)
14. Pacific Blue goat anti-rabbit IgG antibody (Thermo Fisher/Molecular Probes, catalog number: P10994)
15. 35 mm microwell dishes (MatTek, catalog number: P35G-1.5-14-C)
16. Human embryonic kidney 293T cells (American Type Culture Collection, catalog number: CRL-11268)
17. Dulbecco's modified Eagle's medium (DMEM; ThermoFisher/Invitrogen, catalog number: 11965-118)
18. Fetal bovine serum (FBS; Gemini Bio-Products, catalog number: 900-108)
19. Trypsin-EDTA, 0.05% (ThermoFisher/Invitrogen catalog number: 25300054)
20. X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich, catalog number: 06365787001)
21. Paraformaldehyde, 16% aqueous solution (Fisher, catalog number: 50980487)
22. Triton X-100 (Sigma, catalog number: X100-1L)
23. Bovine serum albumin (BSA, Sigma, catalog number: A3059-500G)
24. Itk inhibitor, BMS-509744 (Calbiochem, catalog number: 41-982-05MG)
25. Itk/Btk inhibitor, ibrutinib (SelleckChem, catalog number: S2680)

Equipment

1. Olympus Fluoview FV1000 Confocal Microscope

Software

5. Prism v. 8.0 (GraphPad Software, Inc.; www.graphpad.com)
6. ImageJ (National Institutes of Health; <https://imagej.net/Welcome>)
7. Olympus Fluoview Software (<https://www.olympus-lifescience.com/en/>)

Procedure

A. Construction of expression vectors for BiFC based on Venus.

This procedure is based on our published work with BiFC vectors for lentiviral Nef alleles and the Tec family kinases Btk and Itk (Taraifdar *et al.*, 2014; Li *et al.*, 2020) but can be easily adapted to virtually any combination of interacting protein partners. While our approach uses pcDNA3-based expression vectors that drive strong protein expression in 293T cells, other vectors can be substituted depending on the target cell type and application. Additional details related to primer design, subcloning strategy, and sequences of the final fusion constructs are provided in the [Appendix](#).

1. PCR-amplify the coding sequence for the Venus N-terminal (VN: residues Val² to Asp¹⁷³) and C-terminal (VC: residues Ala¹⁵⁴ to Lys²³⁸) fragments containing the appropriate restriction sites and a stop codon, and subclone the PCR product into the mammalian expression vector, pcDNA3.1(–).
2. PCR-amplify the coding sequence for HIV-1 Nef (SF2) and SIV Nef (mac239) in the same manner and subclone the product into the pcDNA3.1(–)/VN construct.
3. PCR-amplify the full-length human Tec family kinase cDNA clones containing the appropriate restriction sites and a C-terminal V5 epitope tag for ligation in-frame into the pcDNA3.1(–)/VC construct.

B. 293T cell culture and transfection

1. Culture 293T cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C humidified incubator with a 5% CO₂ atmosphere. Use 10 ml DMEM per 10-cm culture dish and maintain cells at a density of 2-5 × 10⁴ cells/cm². When cultures reach 80% confluence, remove the spent medium and split 1:10 by incubating with 1.0 ml 0.05% trypsin-EDTA solution for 1 min at 37°C to generate a single cell suspension. Add 10 ml fresh medium and spin at 500 × g for 5 min. Resuspend the cell pellet in 10 ml fresh medium and transfer 1.0 ml cell suspension to new 10-cm dishes with an additional 10 ml fresh medium. For transfection, seed 2.5 × 10⁵ cells per MatTek plate and culture overnight.
2. Transfect cells with BiFC expression vectors using X-tremeGene 9 DNA transfection reagent according to the manufacturer's protocol. Briefly, mix 3 µL X-tremeGene 9 with 97 µL DMEM, add each BiFC expression plasmid (0.5 µg each VN and VC construct), and incubate the mixture at room temperature for 15 min. After incubation, add the transfection complex dropwise to the MatTek culture plate. Treat the cells with inhibitors or the DMSO carrier solvent (0.1% final concentration) 4 h after transfection as needed.

C. Immunofluorescence staining

1. Forty hours post-transfection, fix the cells by replacing the medium with 2 mL 4% paraformaldehyde and incubate for 10 min at room temperature. Wash cells with 2 ml PBS (pH 7.4) for 5 min with occasional gentle shaking by hand.
2. Permeabilize the cells with 2 mL 0.2% Triton X-100 in PBS for 15 min. Wash cells twice with 2.0 mL PBS for 5 min each.
3. Block the cells with 2 ml 2% BSA in PBS for 1 h at room temperature or overnight at 4°C.
4. Incubate the cells with anti-V5 (kinase tag) and anti-Nef or anti-pTyr (or anti-Btk pY551/anti-Itk pY511) antibodies diluted 1:1,000 in 250 µL PBS with 2% BSA for 1 h at room temperature. Wash cells three times with 2 ml PBS for 5 min each.
5. Incubate the cells with the appropriate secondary antibodies conjugated to Texas Red or Pacific Blue at a dilution of 1:500 or 1:1,000, respectively. Wash the cells three times with 2 mL PBS for 5 min each. Keep cells in 2 mL PBS for imaging.

Note: This protocol was developed for 3-color imaging to allow simultaneous detection of interaction (Venus complementation) and protein expression of each interacting partner (kinase and Nef), or interaction, kinase expression, and kinase activity (overall cellular phosphotyrosine content or activation loop autophosphorylation).

D. Fluorescence imaging and analysis

1. Acquire images using multi-color confocal microscopy with a 60 \times objective using x-y scan mode. On the Olympus FluoView1000, we used the violet laser to detect Pacific Blue (405 nm), the green laser to detect fluorescence complementation (Venus; 543 nm), and the red laser to detect Texas Red (633 nm).
2. Perform single-cell image analysis using the Java-based image processing program, ImageJ, as described under Data analysis.

Data analysis

As an example, the following protocol uses ImageJ to quantitate the mean fluorescence intensities (MFI) of cells stained with anti-phosphotyrosine (anti-pTyr) and anti-Btk antibodies. The ratio of the pTyr to Btk MFIs is then calculated for a minimum of 100 cells as a measure of the kinase activity within the cell population. The same approach can be used to quantitate other MFI ratios such as interaction (BiFC fluorescence) normalized to partner protein expression.

1. File/Open: Open the Btk protein expression and confocal image pair (Figure 2). Minimize the pTyr image and **do not click on it again**. Click on the Btk protein image window.

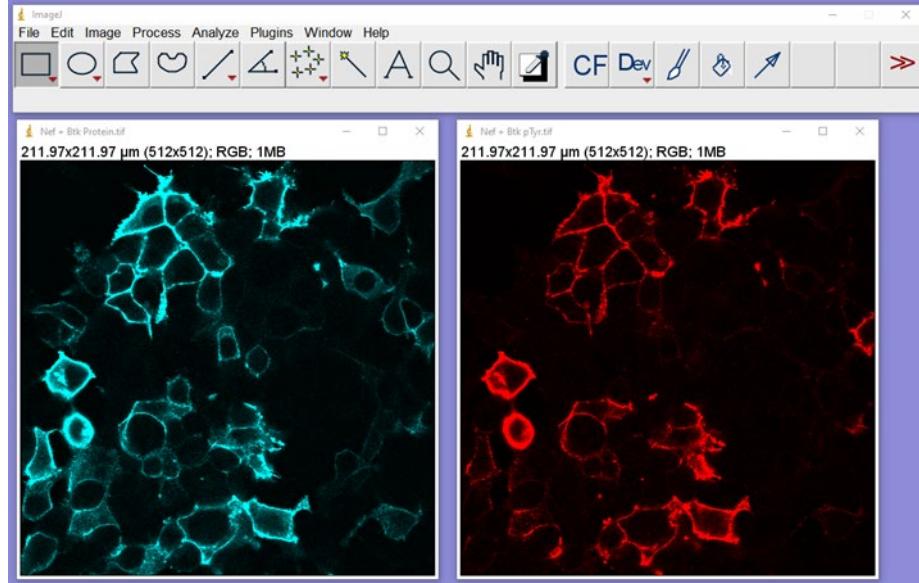


Figure 2. Confocal images of 293T cells stained for Btk protein expression and pTyr opened in ImageJ. Cells stained for Btk protein are shown on the left (cyan), with pTyr shown on the right (red). These images are from cells co-expressing Btk and HIV-1 Nef.

2. Image/Type: Change to 8-bit, which will convert the image to black and white (Figure 3).

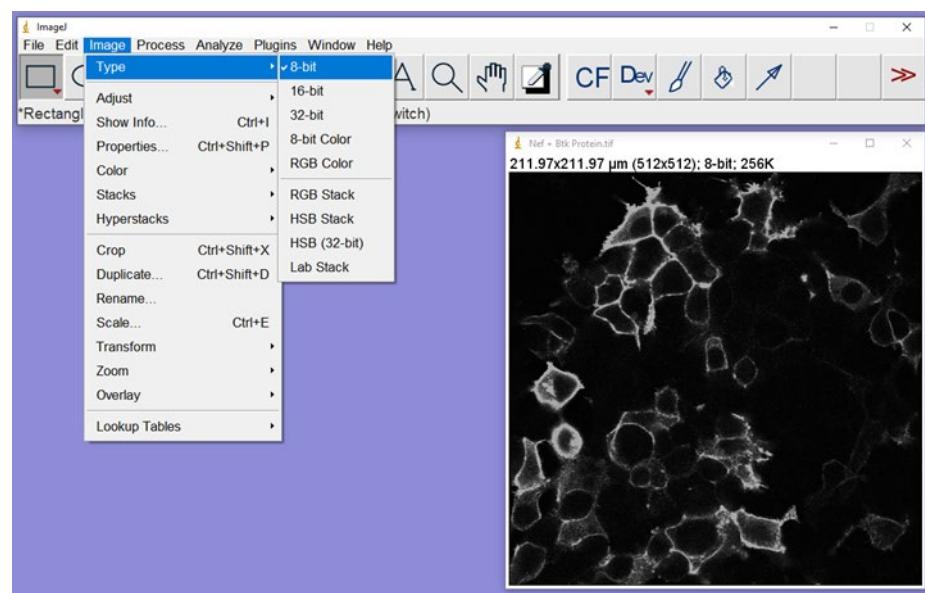


Figure 3. Conversion of Btk protein image to an 8-bit greyscale.

In the control bar, click on Image and then 8-bit, which will convert the image to an 8-bit grey scale image (right).

3. Image/Adjust/Threshold: Set levels by moving the two sliders to set the min/max to include staining but minimize the background and overexposure. Check the “Dark Background” box. Click “Set” and “OK.” Close the Threshold box. The red highlighted areas show the pixels that will be included in the analysis (Figure 4).

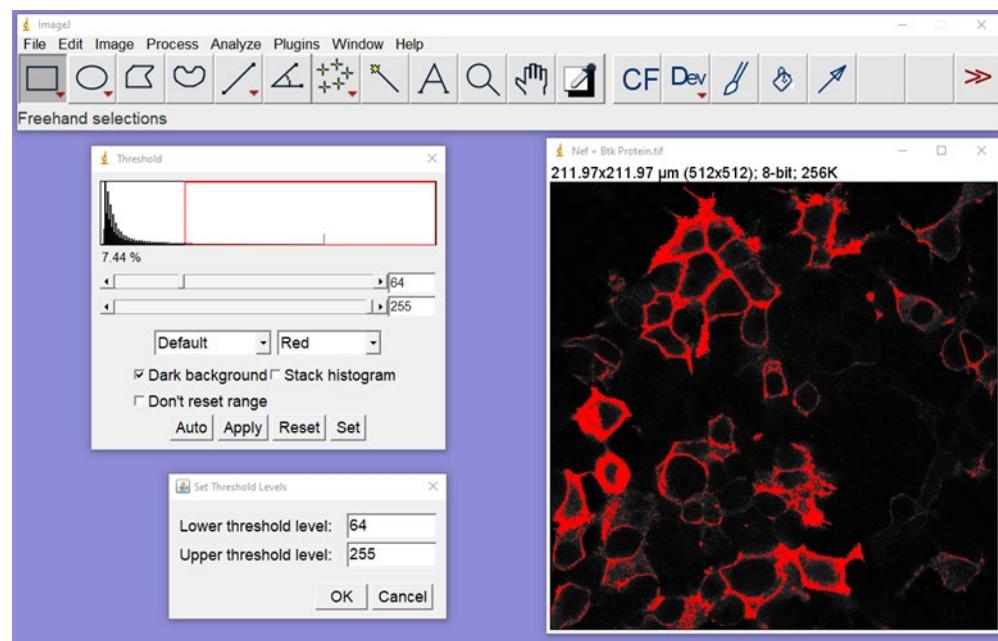


Figure 4. Setting the threshold.

Click on Image in the control bar, select Adjust, and then Threshold. The box on the upper left will appear, and the pixels that will be included in the analysis will be shown in red on the image (right). Click Set; the lower box shown will appear. Click OK to finish.

4. Analyze/Set Measurements: Select these options (Figure 5):
 - a. Area, Min & Max Gray Value, and Mean Gray Value.
 - b. Set the “Redirect to” box to “None.”

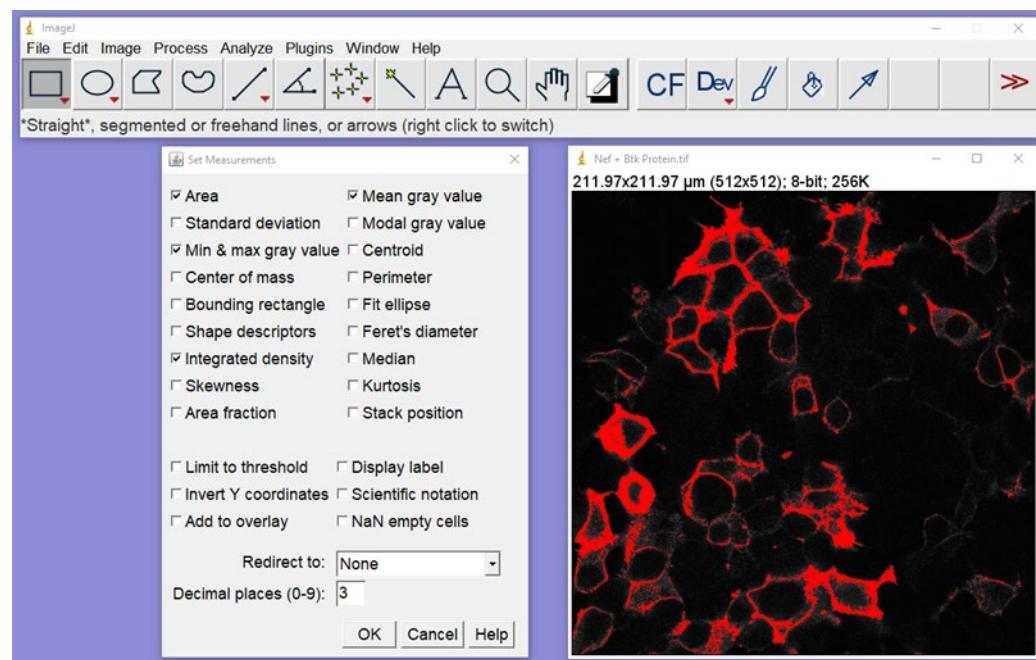


Figure 5. Setting the analysis parameters.

From the control bar, click on Analyze and then Set Measurements; the box shown on the left will appear. Select the parameters shown.

5. Analyze/Analyze Particles (Figure 6):
 - a. Size: Determines the cell area that will be analyzed. Typically, [20-infinity] and [50-infinity] works best for 400× and 100× images of 293T cells, respectively.
 - b. Check the “pixel units” box.
 - c. Circularity: Start with [0.0-1.0] to exclude background specks and noise.
 - d. Show: Choose the “Outlines” option to reveal the location and size of the areas selected for measurement (controlled by adjusting the size/circularity values above).
 - e. Check the “Display Results” box and uncheck the “Clear results” box.
 - f. Click “OK.” Two additional windows will be generated: One (Figure 7) shows outlines of the cell areas used in the analysis of the thresholded image (left), and the second (right) is the results table window containing MFI data (‘Mean’ column).
 - g. Copy the data in the Results window into an Excel file and close the Results window only.

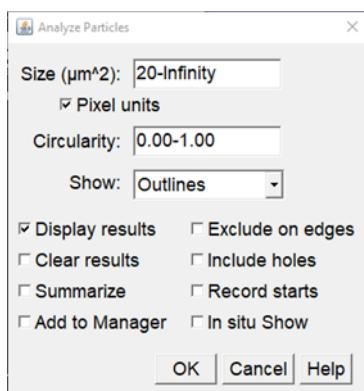


Figure 6. Particle (pixel) analysis settings.

From the control bar, select Analyze and then analyze particle, which will open this box. Select the parameters shown.

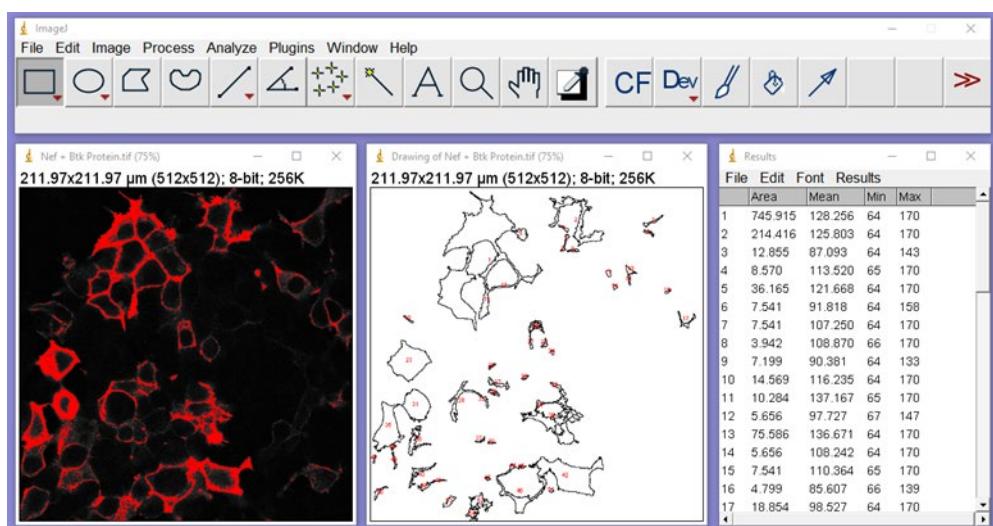
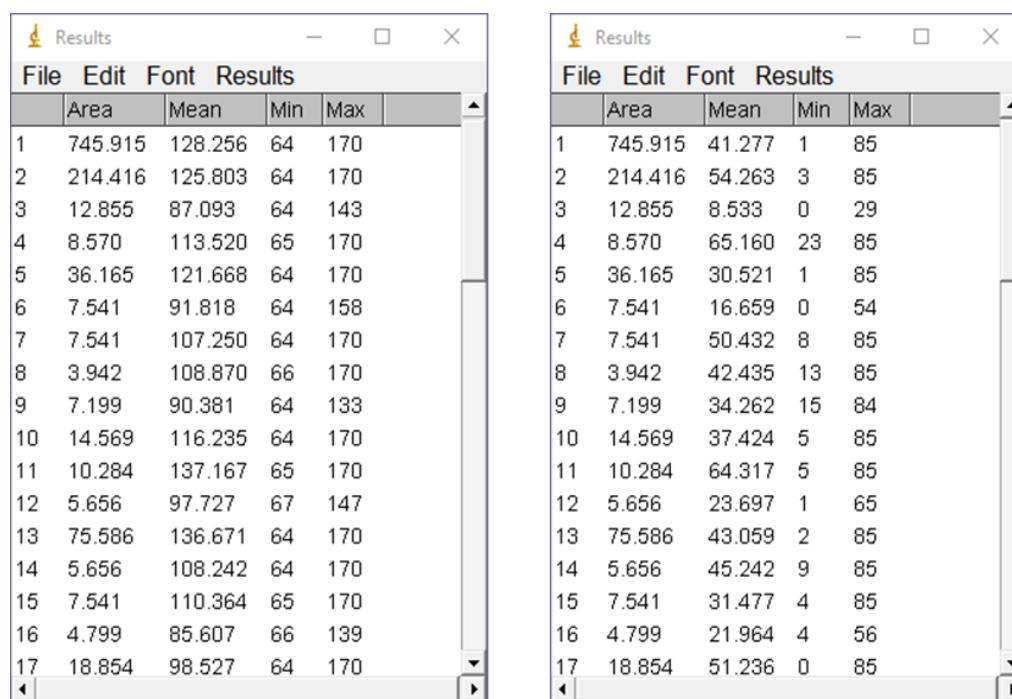


Figure 7. Results of particle analysis.

The center image shows outlines of the areas where pixel intensities have been analyzed and numbered. The table on the right shows the resulting data including the number of the analyzed feature and its area, followed by the mean and minimum and maximum fluorescence intensities. Note that cells will be analyzed individually, as groups, or as fragments, with several fragments from the same cell.

The next step will use the same masks to analyze the anti-pTyr image. Be sure the “Results” box from the protein analysis is closed. Click on the ‘Nef + Btk’ protein box to activate it.

- Analyze/Set Measurements: Set the “Redirect to” box to the corresponding pTyr image file name (previously minimized and not modified) while keeping the other parameters the same. Click “OK.” The selected (outlined) fields established for the Btk protein image are now directly transposed onto the pTyr image and will analyze the pTyr levels relative to the identical cell locations (pixels) provided by the Btk immuno-stain.
- Analyze/Analyze Particles: Use the previous settings and click “OK.” Select all and copy the data in the Results window into the Excel file. The Results windows from the Btk protein and pTyr analyses are shown side-by-side below (Figure 8). Note that the number of rows in each are the same (54; not shown) and the areas used for analysis of each region are also identical. However, the MFI values differ (Mean columns) as expected, since the intensities are different.



The figure displays two identical software windows titled "Results" side-by-side. Both windows have a menu bar with File, Edit, Font, and Results. The main area is a table with columns: Area, Mean, Min, and Max. The left window shows data for the Btk protein, while the right window shows data for pTyr. Both tables contain 17 rows of data, with the last row being a summary or average.

	Area	Mean	Min	Max	
1	745.915	128.256	64	170	
2	214.416	125.803	64	170	
3	12.855	87.093	64	143	
4	8.570	113.520	65	170	
5	36.165	121.668	64	170	
6	7.541	91.818	64	158	
7	7.541	107.250	64	170	
8	3.942	108.870	66	170	
9	7.199	90.381	64	133	
10	14.569	116.235	64	170	
11	10.284	137.167	65	170	
12	5.656	97.727	67	147	
13	75.586	136.671	64	170	
14	5.656	108.242	64	170	
15	7.541	110.364	65	170	
16	4.799	85.607	66	139	
17	18.854	98.527	64	170	

Figure 8. Results of particle analysis of the Btk pTyr image.

The Results tables from the Btk protein (from Figure 7, *left*) and pTyr (*right*) analyses are shown side-by-side for comparison. Note that areas of the features in each image are identical, as are the number of features (54 in each case, not shown). Both conditions must be true for the subsequent calculations to be valid.

8. Using Excel, calculate the ratio of pTyr to Btk mean pixel densities (“Mean” columns).
9. Analyze at least 100 cells per condition, which may require several fields of cells depending on the cell density, size, and magnification used. Significant differences between groups can be analyzed using an unpaired Student’s t-test (GraphPad Prism v.8.0). For added rigor, perform three biological replicates of each experiment.
10. The data can be presented in several different ways using Prism. We prefer showing the results for individual “cells” (really from masks of individual cells or cell fragments as identified by ImageJ) as a series of bars, which gives a view of the distribution of ratios across the cell population. Alternatively, box-and-whisker plots or violin plots can be used. Figure 9 shows the distribution of ratios obtained using the above approach following ImageJ analysis of the results shown in Figure 1.

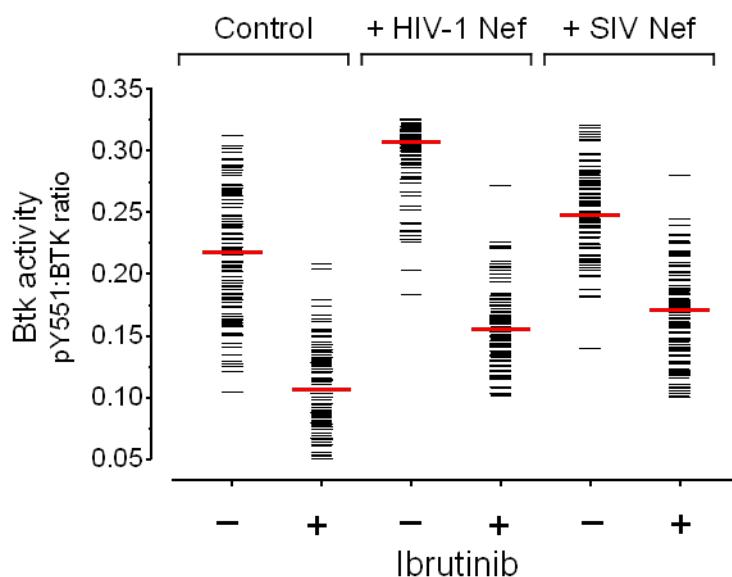


Figure 9. Image analysis of Btk autophosphorylation in the presence and absence of lentiviral Nef proteins.

Mean fluorescence intensities for Btk activation loop autophosphorylation (pY551) and Btk protein expression signals were determined for ≥ 100 cells from each condition using ImageJ and the data shown in Figure 1. The fluorescence intensity ratio (pY551: Btk expression) for each cell (or cell fragment as determined by ImageJ) is shown as a horizontal bar, with the median value indicated by the red bar. Student's t-test shows significant increases in Btk activation loop phosphorylation in the presence of both HIV-1 and SIV Nef ($P < 0.0001$ in each case). When Btk is expressed alone, a wide range of pY551:Btk ratios are observed because the extent of Btk activity increases in a non-linear fashion as the amount of protein increases. When expressed with HIV-1 Nef, note that most of the black bars shift to the top of the stack, consistent with maximal activation of Btk by Nef in almost all the cells imaged. Co-expression with SIV Nef produces a more subtle, albeit statistically significant, shift in the pY551:Btk ratio. Ratios from all three populations shift downward in the presence of the Btk kinase inhibitor, ibrutinib.

Notes

1. Do not allow the dishes to dry out prior to imaging. Drying will seriously affect staining and create false positive artifacts.
2. Protect fluorophores from light to avoid bleaching by wrapping dishes in aluminum foil.
3. Always include positive and negative BiFC pairs as controls, especially when developing the assay for a new protein-protein interaction.
4. 293T cells should be maintained at a low passage number to ensure cell integrity (*i.e.*, less than 10 passages).
5. Keep all confocal image acquisition and ImageJ analysis settings the same throughout an experiment. Results should be reproducible when all conditions are kept constant.
6. When testing a new phosphospecific antibody, titrate a range of antibody concentrations during the staining procedure to identify the optimal concentration.
7. After staining, the cells can be imaged up to 2 days later, and as long as one week in some cases. When storing plates, protect from light and keep at 4°C.
8. During imaging, adjust the laser power to avoid oversaturating the signal in the image, as this may affect subsequent image analysis.

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Competing interests

The authors have no competing interests.

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Yeast Lipid Extraction and Analysis by HPTLC

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Abstract

The diversity of lipid structures, properties, and combinations in biological tissues makes their extraction and analysis an experimental challenge. Accordingly, even for one of the simplest single-cellular fungi, the budding yeast (*Saccharomyces cerevisiae*), numerous extraction and analysis protocols have been developed to separate and quantitate the different molecular lipid species. Among them, most are quite sophisticated and tricky to follow. Herein, we describe a yeast total lipids extraction procedure with a relatively good yield, which is appropriate for subsequent thin-layer chromatography (TLC) or liquid chromatography-mass (LC-MS) analysis. We then discuss the most widely used solvent systems to separate yeast phospholipids and neutral lipids by TLC. Finally, we describe an easy and rapid method for silica gel staining by a Coomassie Brilliant Blue-methanol mixture. The stained lipid species can then be quantitated using imaging software such as ImageJ. Overall, the methods described in this protocol are time-saving and novice-friendly.

Keywords: Lipid extraction, TLC, Lipid staining, Phospholipid, Neutral lipid, Budding yeast

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Background

Lipid research has gained great momentum in recent years, stimulated in part by the interest in lipid-associated disorders in humans, in part by aspirations of biofuel and biopharmaceutical production. Lipids play vital roles in many biological processes, including the formation of bio-membranes, the storage of energy, and numerous signal transduction processes. The budding yeast, *Saccharomyces cerevisiae*, has been an excellent model organism for studying the physiological and pathophysiological roles of lipids at the cellular level (Popa *et al.*, 2016). Primary lipid metabolism is highly conserved between yeast and humans in both the basic biochemical pathways and the regulatory circuitries that link lipid metabolism to energy homeostasis, cell growth, and development. One advantage of using yeast in lipid-related research is that the pathways and molecular compositions are often less complicated than those in mammals, facilitating their scientific investigation. In yeast, PA (phosphatidate acid), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PI (phosphatidylinositol), PS (phosphatidylserine), DAG (diacylglycerols), and their respective lyso-derivatives are the most abundant membrane lipid classes, while PG (phosphatidylglycerol) and CL (cardiolipin) are less abundant. TAG (triacylglycerol) and SE (sterol ester) act as storage lipids. The most abundant yeast sphingolipids consist of IPC (inositolphosphoceramide), MIPC (mannose inositolphosphoceramide), and M(IP)₂C (mannose diinositolphosphoceramide) (Klug and Daum, 2014).

Lipid extraction procedures described in the literature generally share common principles but vary in processing steps. Among these, the most cited Folch method uses CHCl₃/CH₃OH (2:1 v/v) as the solvent system (Folch *et al.*, 1957). Alternate proportions of CHCl₃/CH₃OH or replacement of CHCl₃ with 2-propanol or hexane have also been utilized (Fuchs *et al.*, 2015; Knittelfelder and Kohlwein, 2017a and 2017b). In this protocol, we use CHCl₃/CH₃OH (2:1 v/v) as the solvent for lipid extraction and break yeast cells with glass beads in a mechanical bead mill. We repeat the extraction five times to ensure efficient lipid extraction.

Silica gel is the most widely used stationary phase for chromatographic lipid separation. Impregnated with different substances, modified silica gel can be used to separate various lipid classes. Among the most popular modifications, silver nitrate impregnation has better potential for separating glycerolipids containing unsaturated fatty acyl chains (Dobson *et al.*, 1995); boric acid is primarily used to separate the various isomers of DAG or PL (Ando *et al.*, 2000). Like in other chromatographic techniques, TLC can be classified as “normal” or “reversed” phase based on the polarities of their mobile and stationary phases. Normal phase (polar stationary phase and non-polar mobile phase) chromatography is the standard method to separate lipids of interest that may have different polarities caused by a variation in their head groups (Fuchs *et al.*, 2011). Typically, the size of gel particles for TLC is 10-50 µm. In contrast, the size of the high-performance thin-layer chromatography (HPTLC) gel particles is about 5 µm with narrow distributions (Fuchs *et al.*, 2011), which results in higher separation quality and smaller sample quantity. It is noteworthy that bio-lipids are very complex molecules, and no single separation method is sufficient to separate all lipid species. With the stationary phase chosen, the separating efficiency of TLC or HPTLC mainly relies on the mobile phase. A 0.4% (NH₄)SO₄-impregnated silica gel plate together with chloroform-methanol-acetic acid-acetone-water (40:25:7:4:2 v/v/v/v/v) as the mobile phase can be used to separate phospholipids, lysophospholipids, and SM (sphingomyelin) (Wang and Gustafson, 1992). A chloroform-methanol-water mixture (35:15:2 or 50:40:10 v/v/v) is generally used for the separation of SM; a different proportion, chloroform-methanol-water mixture (65:25:4 v/v/v), can be used to separate phospholipids (Knittelfelder and Kohlwein, 2017a and 2017b). A mobile phase consisting of hexane-diethyl ether-acetic acid (70/30/1 v/v/v) is initially used to separate cholesterol isomers; it can also be used to separate neutral lipids such as TAGs, DAGs, and MAGs (monoacylglycerols). Alternatively, neutral lipids can be separated by TLC silica gel 60 plates and petroleum ether-diethyl ether-acetic acid (32:8:0.8 v/v/v) mixture as the mobile phase (Knittelfelder and Kohlwein, 2017a and 2017b). Overall, it is a trial-and-error process to choose a proper stationary phase and mobile phase based on the characteristics of individual lipid species. In the present protocol, a silica gel 60 plate is used as the stationary phase, and a hexane-diethyl ether-acetic acid (70/30/1 v/v/v) mixture and a chloroform-methanol-water mixture (65:25:4 v/v/v) are used as the mobile phase to separate neutral lipids and phospholipids, respectively. Note that the mobile phase for chromatographic lipid separation is not to be confused with the solvent system for lipid extraction, which is discussed in the preceding paragraph.

After separation, the resulting positions of different lipid species on TLC plates need to be visualized, usually by color reactions with chemical spray reagents. An iodine vapor bath is one of the most widely used methods. The brown iodine color will disappear spontaneously once the plates are removed from the iodine vapor (Palumbo and

Zullo, 1987). It has been reported that iodine can be difficult to remove from highly unsaturated lipids, but in our experience, the brown color fades away too quickly before follow-up steps can be properly performed. Alternative spray reagents include 2,7-dichlorofluorescein, rhodamine 6 G, and primuline. Together with iodine, they belong to non-destructive reagents, with the modification of lipid structures being reversible (Fuchs *et al.*, 2011). 0.2% amino black 10 B in 1 M NaCl and 3.2% H₂SO₄, 0.5% MnCl₂ in 50% ethanol are two destructive reagents with high sensitivity (Fuchs *et al.*, 2011 and 2015; Knittelfelder and Kohlwein, 2017a and 2017b). In this protocol, we use Coomassie Brilliant Blue R-250, a non-destructive reagent first used to stain lipids in 1984 (Nakamura and Handa, 1984). In our experience, the detection sensitivity and durability of Coomassie Brilliant Blue R-250 are better than those of iodine vapor. Conveniently, the staining steps only take 30 min.

Materials and Reagents

1. Pipette tips (Thermo Fisher Scientific, different sizes and types)
2. Centrifuge tube, 5-mL round-bottomed (Sangon Biotech, catalog number: F610888)
3. Cryogenic microtubes, 1.5-mL deep cap, conical-bottomed, sterile (Sangon Biotech, catalog number: F600154)
4. Gel blotting paper (Sangon Biotech, catalog number: F513323)
5. Yeast extracts (OXCID, catalog number: LP0021)
6. Peptone (BD, catalog number: 211677)
7. D-glucose (Sangon Biotech, catalog number: A501991)
8. Yeast nitrogen base without amino acids and ammonium sulfate (Sangon Biotech, BBI, catalog number: A600505)
9. Chloroform (General-Reagent, catalog number: G75915B)
10. Methanol (General-Reagent, catalog number: G75851B)
11. Coomassie Brilliant Blue R-250 (Sangon Biotech, catalog number: CB0037)
12. Hexane (General-Reagent, catalog number: G14153D)
13. Diethyl ether (HUSHI, catalog number: 10009318)
14. Acetic acid (General-Reagent, catalog number: G73562B)
15. Lipid standard: 18:1 DG (1,2-dioleoyl-sn-glycerol) 2 mg/ml chloroform (Avanti, catalog number: 800811C)
16. Lipid standard: Egg PC (L-alpha-phosphatidylcholine) 10 mg/mL chloroform (Avanti, catalog number: 840051C)
17. TLC Silica gel 60 (25 aluminium sheets 20 × 20cm; Merk Millipore, catalog number: 105553)
18. Glass thin-layer chromatography (TLC) developing chambers (100 × 100, generic)
19. Glass beads (40 or 50 mesh, generic)

Equipment

1. Pipettes (Thermo Fisher Scientific, different types)
2. Shaking incubator (CRYSTAL, model: IS-RDH1)
3. Spectrophotometer (Shanghai Jing-Hua, model: 722S)
4. Centrifuge (Thermo, model: SORVALL ST16R)
5. Freeze-dryer/lyophilizer (Labconco, model: Freezone 6 Plus)
6. Analytical scale (Shanghai Heng-Ping, model: FA2104)
7. Drying oven (BOXUN, model: GZX-9146MBE)
8. Bead mill (Shanghai Jing-Xin, model: Tissue lyser-96)
9. Thermomixer (ALLSHENG, model: MSC-100)
10. TLC developing chamber with cover (generic)
11. Gel imaging system (Bio-Rad, model: Gel Doc 721BR05189)

Software

1. ImageJ (<https://imagej.net/Downloads>)

Procedure

A. Cell growth and harvest

1. Grow yeast cells in 100 ml YPD medium (1% yeast extract, 2% peptone, 2% glucose) overnight in a 30°C shaking incubator (250 rpm) until the optical density (OD600) of the culture is about 0.7-1.0.
2. Harvest the cells by centrifugation, $10,000 \times g$ for 5 min at room temperature. Remove the supernatant completely and discard.
3. Freeze-dry the cells in a freeze-dryer and store at -80°C.

B. Total lipid extraction

1. For each freeze-dried sample, transfer 0.25 mg into a 1.5-mL cryogenic microtube.
2. Add 200 μ L washed glass beads to each sample tube.
3. Add 500 μ L chloroform-methanol (2:1, v/v) to each sample tube. Screw on the Teflon-sealed cap tightly.
4. Place the sample tubes in a bead mill and perform mechanical shearing at maximum speed for 5 min at room temperature. Repeat the mechanical lysis twice; rearrange tube locations in-between to avoid uneven cell lysis (Step 1 in Figure 1).
5. Centrifuge the cell lysates at room temperature, $10,000 \times g$ for 5 min. After centrifugation, cell debris will be sandwiched between the supernatant and the glass beads (Step 2 in Figure 1). Transfer the supernatant to new tubes for collection (Step 3 in Figure 1).
6. Repeat the chloroform-methanol extraction procedure (Steps 2-6 in Figure 1) five times.
7. Merge the supernatant collections of each sample in a 5-ml tube and evaporate on a compact thermomixer at 60°C overnight with the lid off. This step needs to be performed in a fume hood. After evaporation, membrane-like substances will be left at the bottom of the tubes.

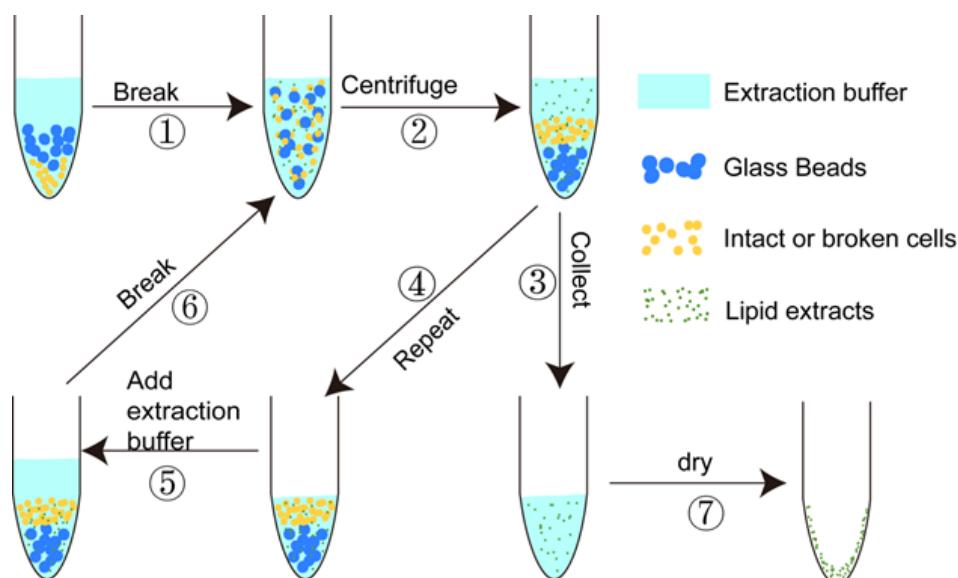


Figure 1. Schematic depiction of the lipid extraction procedure

C. Lipid analysis by TLC

1. Dissolve the lipid extracts in 100-500 μ L chloroform.
2. Prepare the solvent mixtures:
 - a. For separation of phospholipids, prepare a chloroform-methanol-H₂O (65:25:4, v/v/v) mixture (Knittelfelder and Kohlwein, 2017a and 2017b).
 - b. For separation of neutral lipids, prepare a hexane-diethyl ether-acetic acid (70:30:1 v/v/v) mixture (Fuchs *et al.*, 2015).
3. Add the mobile phase to glass TLC developing chambers. Saturate the chamber atmosphere with folded blotting paper for more than 30 min.
4. Activate the silica gel 60 plates at 100°C in an oven for 30 min. Draw a fine spotting line with a pencil 2 cm from the bottom of the TLC plate.
5. Spot 5-20 μ L dissolved samples on the activated silica gel plates carefully with 10- μ l pipette tips. Keep the spacing between these spots larger than 0.5 cm. In addition, spot 0.2-1 μ g lipid standards (DAG or PC) to distinguish the complex bands of each sample and to monitor the developing and staining steps.
6. Develop each plate in a solvent chamber until the solvent front reaches the top of the plate. To maintain a solvent-saturated atmosphere in the chamber, do not open the lid while the plate is developing.
7. Dry the plates in a fume hood for 20 min at room temperature.
8. Stain the dried plates with a 0.03% Coomassie Brilliant Blue R-250 solution containing 20% methanol for 15 min, and subsequently de-stain in 20% methanol for 10 min (Nakamura and Handa, 1984).
9. Dry the plates under room temperature for 1 h and take photos by a gel imaging system (Figure 2).
10. Analyze the grayscale of each lipid band using ImageJ (Steps: Open an image file in ImageJ – go to Analyze – gels – Select First Line – Select Next Line – Wand (tracing) Tool – Plot Lanes).

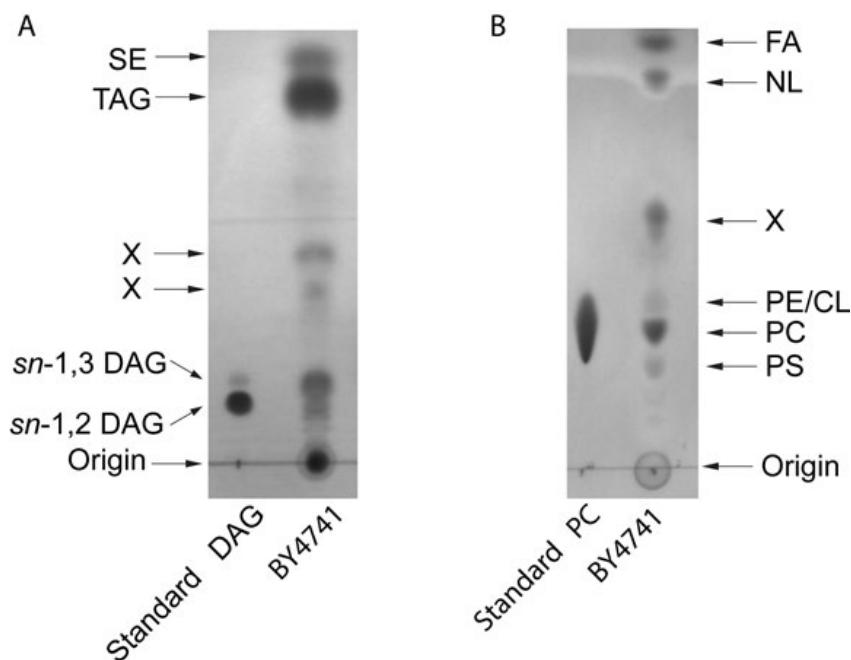


Figure 2. Exemplary HPTLC separation of yeast lipids.

A. Neutral lipids: the solvent system was hexane-diethyl ether-acetic acid (70:30:1 v/v/v). DAG, diacylglycerol; TAG, triacylglycerol; SE, steryl esters. B. Polar lipids: the solvent system was chloroform-methanol-H₂O (65:25:4, v/v/v). FA, fatty acids; NL, neutral lipids; PE, phosphatidylethanolamine; CL, cardiolipin; PC, phosphatidylcholine; PS, phosphatidylserine. X, unidentified. Lipids were stained with Coomassie Brilliant Blue R-250.

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Competing interests

The authors declare no competing financial interests

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PCR-mediated One-day Synthesis of Guide RNA for the CRISPR/Cas9 System

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Abstract

Nowadays, CRISPR (clustered regularly interspaced short palindromic repeats) and the CRISPR-associated protein (Cas9) system play a major role in genome editing. To target the desired sequence of the genome successfully, guide RNA (gRNA) is indispensable for the CRISPR/Cas9 system. To express gRNA, a plasmid expressing the gRNA sequence is typically constructed; however, construction of plasmids involves much time and labor. In this study, we propose a novel procedure to express gRNA via a much simpler method that we call gRNA-TES (gRNA-transient expression system). This method employs only PCR, and all the steps including PCR and yeast transformation can be completed within 1 day. In comparison with the plasmid-based gRNA delivery system, the performance of gRNA-TES is more effective, and its total time and cost are significantly reduced.

Keywords: CRISPR/Cas9, Genome editing, Guide RNA, PCR-based, Yeast

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Background

Developing genome editing techniques is one of the central issues of genome science. For the past decade, the CRISPR/Cas9 system has contributed to easier and more precise genome editing as compared with previously developed techniques such as ZFN (zinc finger nuclease) and TALEN (transcription activator-like effector nuclease). For successful CRISPR/Cas9 engineering, design, expression, and delivery of the guide RNA (gRNA) components are the key factors (Stovicek *et al.*, 2017). For prokaryotes like *Escherichia coli* and eukaryotes such as *Saccharomyces cerevisiae*, the most commonly employed method for expressing gRNA is to use a plasmid (Jiang *et al.*, 2013; Li *et al.*, 2015; DiCarlo *et al.*, 2013; Bao *et al.*, 2015; Jakociunas *et al.*, 2015a and 2015b); however, plasmid construction, including cloning steps for necessary components, is laborious, costly, and time-consuming. To express gRNA more simply, in this study we developed a method that we call gRNA-transient expression system (gRNA-TES), where gRNA is expressed from the PCR product. gRNA-TES is very fast and effective: it takes only 5-6 h to complete the whole process, including preparation of PCR products for expression of gRNA in yeast cells and yeast transformation. By contrast, it takes at least 3-4 days to construct a plasmid expressing gRNA including verification. As expected, when applied to replacement of desired chromosome regions in yeast, gRNA-TES effectively replaces single and multiple chromosomal regions (Easmin *et al.*, 2019 and 2020). Therefore, we believe that gRNA-TES will be useful for other types of genome editing including segmental deletion, duplication, and splitting of chromosomes. Lastly, gRNA-TES is effective in yeast; therefore, it should be emphasized that gRNA-TES may also be efficacious in other organisms if suitable gene promoters are incorporated.

Materials and Reagents

1. 0.1-10 µL pipette tips (BMBio, catalog number: BMT-10GXLR)
2. 20-200 µL pipette tips (BMBio, catalog number: BMT-200R)
3. PCR tubes (Axygen, catalog number: SKPCR)
4. p426-SNR52p-gRNA.CAN1.Y-SUP4t (Addgene, catalog number: 43803)
5. p414-TEF1p-Cas9-CYC1t (Addgene, catalog number: 43802)
6. *Escherichia coli* DH5α competent cells (NIPPON GENE, catalog number: 316-06233)
7. DNA, MB-grade from fish sperm (Roche Diagnostics, catalog number: 11467140001)
8. KOD plus neo (TOYOBO, catalog number: KOD-401)
9. 2 mM dNTP solution (included with KOD plus neo)
10. 25 mM magnesium sulfate (MgSO₄) (included with KOD plus neo)
11. Oligonucleotide Primer Fw A for construction of fragment A (5'-GTTCGAAACTTCTCCGCAGT GAAAGATAATGATCN₂₀GTTTAGAGCTAGAAATAGCAAG-3') (synthesized by Hokkaido System Science, Japan) (N₂₀ represents the 20-nt upstream sequence of the target PAM sequence)
12. Oligonucleotide primer Rv A for construction of fragment A (5'- ACTCACAAATTAGAGCTTCA -3') (synthesized by Hokkaido System Science, Japan)
13. Oligonucleotide primer Fw B for construction of fragment B (5'- CGAACGACCGAGCGCAGCGA-3') (synthesized by Hokkaido System Science, Japan)
14. Oligonucleotide primer Rv B for construction of fragment B (5'- TTATCTTCACTGCGGAGAAGTTTCAAC-3') (synthesized by Hokkaido System Science, Japan)
15. Ex Taq® DNA polymerase (TaKaRa, catalog number: RR001A)
16. 10× Ex Taq buffer (Mg²⁺ plus) (included with Ex Taq® DNA Polymerase)
17. 2.5 mM each dNTP mix (included with Ex Taq® DNA Polymerase)
18. Ampicillin (Nacalai tesque, catalog number: 02739-74)
19. Lithium acetate dihydrate (Sigma-Aldrich, catalog number: L6883-250G)
20. Polyethylene glycol 3,350 (Sigma-Aldrich, catalog number: P4338-500G)
21. Sodium hydroxide (NaOH) (FUJIFILM Wako Pure Chemical Corporation, catalog number: 192-15985)

22. Control primer 1 for amplifying the *CNE1* region (5'-TCACAGGGTCGATTGCAAGG-3') (synthesized in Hokkaido System Science, Japan)
23. Control primer 2 for amplifying the *CNE1* region (5'-CTGGTGGTCAGTGCCATCT-3') (synthesized in Hokkaido System Science, Japan)
24. Oligonucleotide primer 1 for checking replacement (Use the 200-176 nt upstream sequence of the target region)
25. Oligonucleotide primer 2 for checking replacement (Use the 66-90 nt downstream reverse sequence of the target region)
26. Prime Star Max Premix 2× (TaKaRa, catalog number: R045A)
27. Agar (FUJIFILM Wako Pure Chemical Corporation, catalog number: 010-08725)
28. Gene Ladder Wide 2 (Nippon Gene, catalog number: 310-06971)
29. Glucose (FUJIFILM Wako Pure Chemical Corporation, catalog number: 043-31163)
30. Yeast nitrogen base without amino acids (BD, Difco, catalog number: DF0919-15-3)
31. Peptone (BD, BactoTM, catalog number: 211677)
32. Yeast extract (BD, BactoTM, catalog number: 288620)
33. Adenine HCL (FUJIFILM Wako Pure Chemical Corporation, product code: 016-00802)
34. Synthetic Complete (SC) medium (see Recipes)
35. YPDA medium (see Recipes)

Procedure

A. Designing the 20-nt gRNA target sequence

To design the gRNA target sequence, it is essential to have an appropriate PAM sequence. To design a target sequence with a PAM sequence for Cas9 cleavage (5'-NGG-3' or 5'-CCN-3' for opposite strand) close to the target region, we use the free software CRISPRdirect (<https://crispr.dbcls.jp/>). For *S. cerevisiae*, we select the *S. cerevisiae* representative strain S288C and paste 50-100 nt of the sequence located near the target site into the software. Then, CRISPRdirect quickly outputs the appropriate 20-nt gRNA target sequence with a PAM sequence.

B. Preparation of PCR fragments A and B

We use p426-SNR52p-gRNA.CAN1.Y-SUP4t as a common template plasmid to construct a PCR fragment (fragment C) expressing gRNA harboring the designed 20-nt target sequence. It contains the yeast promoter *SNR52*, which is responsible for expressing gRNA in yeast cells. If you want to express gRNA in another organism, you will need to use a template plasmid harboring a suitable promoter sequence obtained from that respective organism to prepare fragment C. Here, we explain the case in which gRNA is expressed from fragment C in a *S. cerevisiae* host. Before preparing fragment C, fragments A and B must be synthesized in two separate steps by first-round PCR.

Fragment A: For preparation of fragment A, design the forward primer (Fw A) to include 35 nt of the sequence from part of the *SNR52* promoter region (3,855-3,889 nt) of the template plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (full sequence is available at Addgene repository, <https://www.addgene.org/43803/sequences/>), followed by the 20-nt gRNA target sequence, and a further 23 nt of the sequence encoding the 5' part of the gRNA scaffold from the template plasmid (3,910-3,932 nt) (Figure 1A). Design the reverse primer sequence (Rv A) based on 5,001-5,020 nt of the reverse sequence (5'-ACTCACAAATTAGAGCTTCA-3') of the template plasmid.

Fragment B: Since fragment A does not contain the full sequence of the *SNR52* promoter and it is essential to have this complete region for proper expression of gRNA, we need to incorporate the whole *SNR52* promoter region with fragment A via fragment B. Therefore, we use a forward primer (Fw B) consisting of a 20-nt

sequence (5'-CGAACGACCGAGCGCAGCGA-3') and a reverse primer (Rv B) consisting of a 30-nt sequence (5'-TTTATCTTCACTGCGGAGAAGTTCGAAC-3') of the template plasmid (3,301-3,320 nt and 3,855-3,884 nt in reverse sequence, respectively) to synthesize the whole *SNR52* promoter sequence as fragment B (Figure 1A). The complementary sequence of the last 30 nt in fragment B is the same as the first 30 nt of fragment A in order to allow fragments A and B to be annealed in the second-round (overlap) PCR.

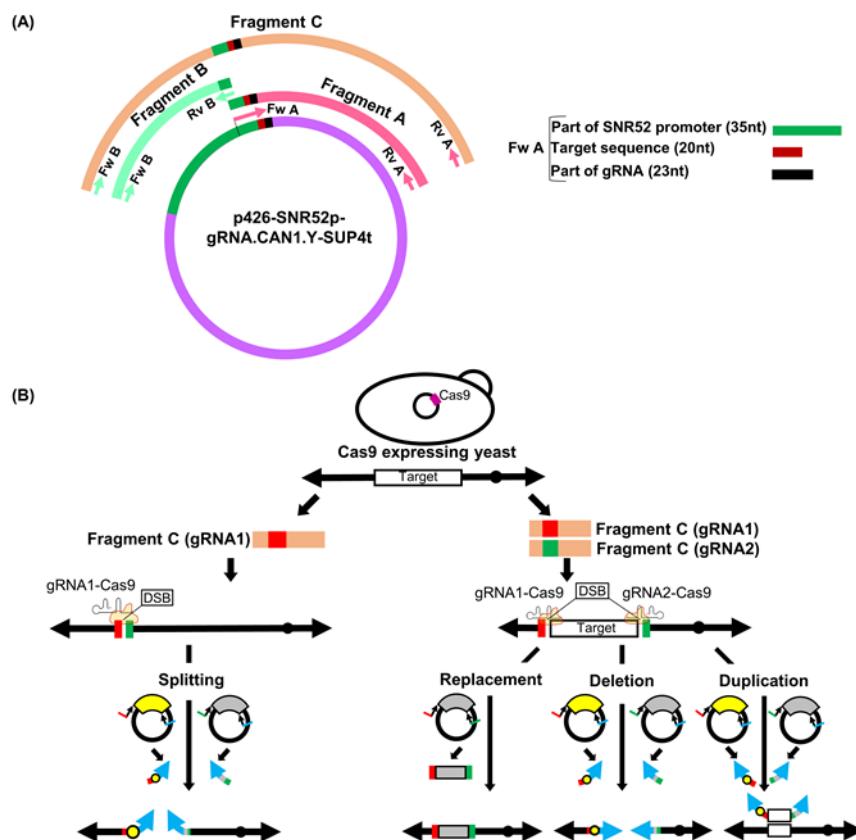


Figure 1. Overview of gRNA-TES.

A. In gRNA-TES, we prepare three fragments, namely fragments A, B, and C, by PCR. Fragments A and B are prepared by a first PCR using plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (DiCarlo *et al.*, 2013) as a common template. Next, fragment C, for expressing the gRNA sequence, is synthesized by a second PCR (overlap PCR) using fragments A and B as templates. In gRNA-TES, a new fragment A is always needed because it contains the 20-nt unique gRNA target sequence. The 20-nt unique gRNA target sequence is changed depending on the target region. By contrast, fragment B is solely the *SNR52* promoter region; therefore, it is not necessary to synthesize a new fragment B with a different sequence every time. Using this protocol, it is not possible to design a primer sequence that can amplify the target sequence along with the whole *SNR52* promoter region since it is difficult to chemically synthesize a good-quality oligonucleotide with more than 100 nucleotides. Thus, we cannot amplify fragment C by a single round of PCR. B. gRNA-TES can be used for various genome manipulations such as chromosome splitting, replacement, deletion, and duplication. Only one fragment C, which delivers one gRNA, is necessary for splitting. Double-strand break (DSB) significantly increases the frequency of homologous recombination between the target sequence (red and green box) on the chromosome and its corresponding DNA modules synthesized from plasmids p3121 (Sugiyama *et al.*, 2005) and pSJ69 (Easmin *et al.*, 2019). As a consequence, a high frequency of splitting is thought to occur. Two kinds of fragment C, which deliver two independent gRNAs, are necessary for replacement, deletion, and duplication. For replacement, we prepare one DNA module harboring an appropriate marker gene (here *Candida glabrata* *LEU2*, *CgLEU2*) flanked with the target homology sequences using plasmid pSJ69 (Easmin *et al.*, 2019). For

segmental deletion and duplication, we need two DNA modules harboring the target homology sequences: one should have a centromere, and the other should contain the marker gene. In our experiments, we use p3121 (Sugiyama *et al.*, 2005) harboring *CEN4* and pSJ69 harboring *CgLEU2* as templates to synthesize the DNA modules. After successful double-strand break, a high frequency of replacement, deletion, and duplication is expected. In panel B, the black circle on the chromosome denotes the native centromere; the yellow circle on the DNA modules denotes artificially supplied *CEN4*. The blue arrow indicates the artificially supplied telomere; the gray box represents *CgLEU2*. The plasmids containing yellow- and gray-colored curved boxes are p3121 and pSJ69, respectively.

First-round PCR reaction

10× Ex Taq Buffer	5 µL
2.5 mM dNTP solution	4 µL
Template plasmid (approximately 50 ng)	1 µL
Oligonucleotide primer 1 (15 pmol)	1.5 µL
Oligonucleotide primer 2 (15 pmol)	1.5 µL
Ex Taq® DNA Polymerase	0.25 µL
Water	36.75 µL
Total	50 µL
Pre-denaturation: 94°C, 2 min	
Denature: 98°C, 10 s	
Annealing: 55°C, 30 s	
Extension: 68°C, 2 min	
	30 cycles

C. Preparation of fragment C by overlap PCR

To prepare fragment C, a 100-fold dilution ($\times 1/100$) of fragment A is mixed with the same dilution of fragment B and used as a template for the second-round PCR (overlap PCR) to generate fragment C with forward primer (Fw B) 5'-CGAACGACCGAGCGCAGCGA-3' and reverse primer (Rv A) 5'-ACTCACAAATTAGAGCTTCA-3'.

Second-round PCR reaction

10× Ex Taq Buffer	5 µL
2.5 mM dNTP solution	4 µL
Template ($\times 1/100$ fragment A)	0.5 µL
Template ($\times 1/100$ fragment B)	0.5 µL
Oligonucleotide primer 1 (15 pmol) 5'-CGAACGACCGAGCGCAGCGA-3'	1.5 µL
Oligonucleotide primer 2 (15 pmol) 5'-ACTCACAAATTAGAGCTTCA-3'	1.5 µL
Ex Taq® DNA Polymerase	0.25 µL
Water	36.75 µL
Total	50 µL
Pre-denaturation: 94°C, 2 min	
Denature: 98°C, 10 s	
Annealing: 58°C, 30 s	
Extension: 68°C, 3 min	
	30 cycles

Note: We recommend using Ex Taq® DNA polymerase for gRNA-TES, especially for the second-round PCR (overlap PCR). Although we tested various DNA polymerases, we observed that those DNA polymerases frequently produced multiple unexpected bands, especially during the overlap PCR used to prepare fragment C.

Application of gRNA-TES to various genome manipulations

gRNA-TES has a variety of applications from splitting to segmental replacement, deletion, and duplication of chromosomes (Figure 1B). Depending on the genome engineering technique, up to two kinds of fragment C delivering two types of gRNA are necessary. After DSB, the frequency of splitting, replacement, deletion, and duplication is expected to be increased.

1. Preparation of DNA modules

To split, delete, replace, or duplicate target chromosomal regions, it is necessary to incorporate DNA modules into gRNA-TES. The type of DNA module that is constructed depends upon the purpose (splitting, deletion, replacement, or duplication) of genome editing. For replacement, only one DNA module is sufficient, while for splitting, deletion, or duplication, two DNA modules are needed (Figure 1B). For **replacement**, design and purchase oligonucleotide primers to amplify any genetic marker flanked with the homology sequence corresponding to the first and last 30 bp of the target region. For **splitting** and **deletion**, design forward primers to amplify the centromere or marker gene flanked with 50 bp of the homology sequence corresponding to the upstream or downstream sequence of the target splitting and deletion point. For **duplication**, design forward primers to amplify the marker gene or centromere flanked with the homology sequence corresponding to the first or last 50 bp of the target region. To include a telomere seed sequence in all DNA modules for splitting, deletion, and duplication, we use a common reverse primer including the 5'-CCCCAACCCCAACCCCAACCCCAACCCCAA-3' sequence. You can use any template plasmid depending on the purpose of genome editing. We use pSJ69 (Easmin *et al.*, 2019) and p3121 (Sugiyama *et al.*, 2005) to synthesize DNA modules. These plasmids have the same background because they were constructed from the same plasmid pUG6 (Güldener *et al.*, 1996) and are available upon request. For splitting, deletion, or duplication, it is necessary to select an appropriate plasmid as a template for PCR so that the newly generated chromosomes contain only one centromere.

PCR reaction

10× KOD plus neo buffer	5 µL
2 mM dNTP solution	5 µL
25 mM MgSO ₄	3 µL
Template plasmid	1 µL

Primers for Replacement

Oligonucleotide primer 1 (15 pmol) 5'-N30FGGCCGCCAGCTGAAGCTTCG-3'	1.5 µL (N30F represents the first 30-bp sequence of the target region)
Oligonucleotide primer 2 (15 pmol) 5'-N30LAGGCCACTAGTGGATCTGAT-3'	1.5 µL (N30L represents the last 30-bp reverse sequence of the target region)

Primers for Splitting and Deletion

Forward primer (15 pmol)	1.5 μ L (N50U/50D represents the 50-bp upstream or downstream (reverse) sequence of the target region)
5'- N50U/50DGGCCGCCAGCTGAAGCTTCG- 3'	
Reverse primer (15 pmol)	1.5 μ L

Reverse primer (15 pmol)
5'

CCCCAACCCAAACCCCCAACCCCCAACCC
CAACCCCCAAGGCCACTAGTGGATCTG
AT-3'
Primers for Duplication

Reverse primer (15 pmol)	1.5 μ L
5'-	
CCCCAACCCCAACCCCAACCCCAACCC	
CAACCCCAAAGGCCACTAGTGGATCTG	
AT-3'	
KOD plus neo	1 μ L
Water	32 μ L
Total	50 μ L
Pre-denaturation: 94°C, 2min	
Denature: 98°C, 10 s	
Annealing: 55°C, 30 s	
Extension: 68°C, 2 min	
	30 cycles

2. Yeast transformation

- a. Prepare in advance a yeast strain, for example, SJY30 (*MATa ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63* harboring plasmid p414-TEF1p-Cas9-CYC1t [DiCarlo *et al.*, 2013]), that expresses codon-optimized Cas9 by the introduction of p414-TEF1p-Cas9-CYC1t.
- b. Cultivate the strain overnight in YPDA liquid medium.
- c. Inoculate a fresh 5-ml aliquot of YPDA liquid medium with yeast cell pre-culture to an initial OD₆₀₀ of approximately 0.2-0.3. Then incubate with a shaking speed of 140 rpm at 30°C until the OD₆₀₀ reaches 0.8-1.0 (about 4-6 h).
- d. Mix the appropriate DNA modules and gRNA-expressing fragment C (*e.g.*, we need two kinds of fragment C [Figure 1B] to target both edges of the target region and one DNA module to replace chromosomal regions) and perform transformation using the conventional LiAc/PEG method (Gietz and Schiestl, 2007).
- e. The aim of this protocol is to provide users with a procedure that is as easy as possible at every step, including colony PCR described in the next section. Therefore, the protocol was developed without measuring the DNA concentration of PCR products. For routinely performed yeast transformation, we consistently use 12 μ L DNA module and 11 μ L each fragment C PCR reaction mixture to replace chromosomal regions, since we have obtained sufficient transformants using such amounts (*i.e.*, 12 μ L + 11 μ L + 11 μ L = 34 μ L) of PCR reaction mixture. Furthermore, the whole PCR reaction mixture can be directly used for yeast transformation without purification.
- f. After yeast transformation, suspend the cells in 100 μ L sterilized water, spread the whole suspension onto one or two selection plates, and incubate at 30°C for 2-3 days.

3. Confirmation of the expected chromosomal change by colony PCR and subsequent agarose gel electrophoresis

- a. To make PCR-grade genomic DNA, take a small amount of cells from each colony and suspend in 10 μ L 0.02 M NaOH solution. Heat at 98°C for 10 min in a heat block and then transfer to ice.
- b. As an example, we describe how to confirm transformants obtained from a replacement experiment here. Design and purchase oligonucleotide primers for colony PCR. You can use any sequence from the upstream and downstream sequences of the target region, but we recommend designing the primer in such a way that the final PCR product will be <2 kb, since generating a larger PCR product may be problematic during colony PCR. For oligonucleotide primer 1, use a 200-176 nt upstream sequence of the target region and for oligonucleotide primer 2, use a 66-90 nt downstream reverse sequence of the target region. Since we use the *CgLEU2* marker gene for replacement and the size of *CgLEU2* is 1685 bp, after successful replacement of the target region, the size of the PCR product will be 200 bp + 1685 bp + 90 bp = 1975 bp (Figure 2). For example, to check the replacement of a 500-kb region in Chromosome 4 (coordinate number 494271-994270), design oligonucleotide primer 1 based on the 494071-494095 nt sequence (5'-CATATCAGTGTCTTCATCTTCATGA-3') and oligonucleotide primer 2 based on the 994,336-994,360 nt reverse sequence (5'-TAGTGGATACGCAGGACGTGTTATC-3') of Chromosome 4. In addition, we recommend

designing control primers to check whether the PCR reaction is proceeding well. Any genomic region may be amplified as a control; we amplify the *CNE1* region of Chromosome 1 as an internal control. Control primer 1 is based on the 211-230 nt sequence (5'-TCACAGGGTCGATTGCAAGG-3') and control primer 2 on the 861-880 nt reverse sequence (5'-CTGGTGGTTCAGTGCCATCT-3') of the *CNE1* region. If the *CNE1* gene is properly amplified, you will obtain a 670-bp PCR product.

c. PCR reaction

Prime Star Max Premix 2×	12.5 µL
Template genomic DNA	0.5 µL
Control primer 1 (7.5 pmol) 5'-TCACAGGGTCGATTGCAAGG-3'	0.75 µL
Control primer 2 (7.5 pmol) 5'-CTGGTGGTTCAGTGCCATCT-3'	0.75 µL
Oligonucleotide primer 1 (7.5 pmol) (Use the 200-176 nt upstream sequence of the target region)	0.75 µL
Oligonucleotide primer 2 (7.5 pmol) (Use the 66-90 nt downstream (reverse) sequence of the target region)	0.75 µL
Water	9 µL
Total	25 µL
Denature: 98°C, 10 s	
Annealing: 55°C, 5 s	
Extension: 72°C, 10 s	
	}
	30 cycles

Note: You can use any DNA polymerase for colony PCR. However, since our intention is to make this protocol as fast as possible, we use Prime Star Max Premix 2×, which requires an extension time of only 5 s/kb..

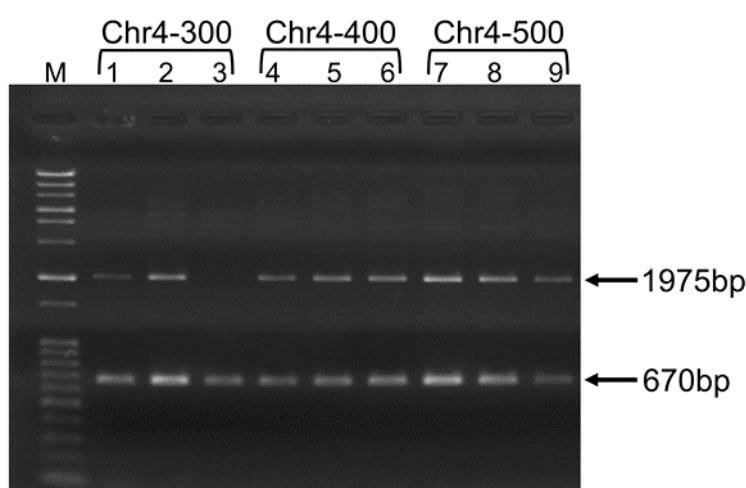


Figure 2. Typical agarose gel electrophoresis results for chromosome replacement analysis by gRNA-TES.

Lanes 1 to 9 represent independent transformants. M represents size markers (Gene Ladder Wide 2, Nippon Gene, Toyama, Japan). Lanes 1 to 3 represent transformants obtained from replacement of a 300-kb region; lanes 4 to 6 represent transformants obtained from replacement of a 400-kb region; and lanes 7 to 9 represent transformants obtained from replacement of a 500-kb region of Chromosome 4. Almost

all transformants (except those in lane 3) showed the expected replacement of the respective chromosomal region and yielded the 1975-bp band. The internal control band (670 bp) was also observed in all transformants.

Data analysis

The number of transformants varies in each experiment. For example, when we targeted the replacement of smaller chromosomal regions of, for example, 150 kb and 200 kb, we obtained 263 and 287 transformants, respectively, in a single transformation (Easmin *et al.*, 2019). When we targeted 300-kb, 400-kb, and 500-kb regions, we obtained, respectively, 51, 43, and 103 transformants in a single transformation. We tested six transformants for replacement of each of the 150-kb and 200-kb regions, and 5 transformants for each of the 300-kb, 400-kb, and 500-kb regions. The frequencies of expected replacement of the 150-kb, 200-kb, 300-kb, 400-kb, and 500-kb regions were 100%, 66.6%, 80%, 100%, and 100%, respectively (Easmin *et al.*, 2019). By contrast, a maximum of 16.6% expected frequency was observed for replacement of the 150-kb region when gRNA-TES was not applied; for replacement of the other chromosomal regions, no correct transformants were obtained when gRNA-TES was not employed.

Recipes

1. Synthetic complete (SC) medium

2% glucose
0.67% yeast nitrogen base without amino acids (*e.g.*, BD Difco)
Note: 0.2% dropout mix containing all amino acids and nucleic acid bases lacking specific amino acids can be used for auxotrophic selection.
For the plate assay, 2% agar is added

2. YPDA medium

2% glucose
2% peptone (*e.g.*, BD BactoTM)
1% yeast extract (*e.g.*, BD BactoTM)
0.004% adenine HCl (FUJIFILM Wako Pure Chemical Corporation)

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Cell-free Translation: Preparation and Validation of Translation-competent Extracts from *Saccharomyces cerevisiae*

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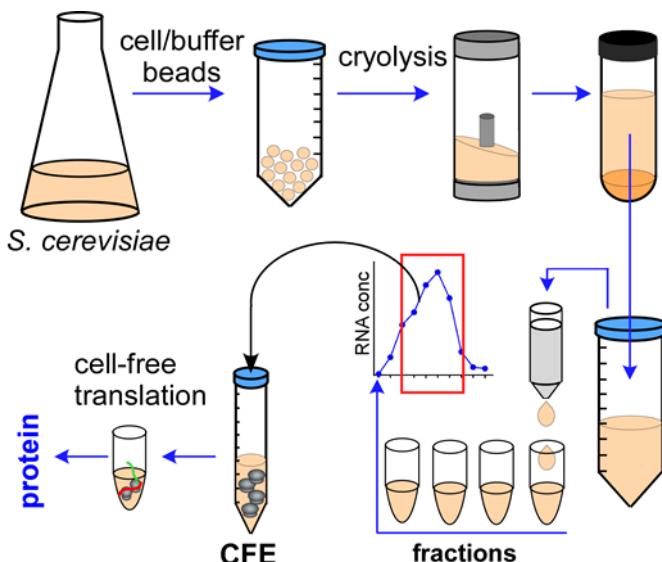
Abstract

Cell-free translation is a powerful technique for *in vitro* protein synthesis. While cell-free translation platforms prepared from bacterial, plant, and mammalian cells are commercially available, yeast-based translation systems remain proprietary knowledge of individual labs. Here, we provide a detailed protocol for simple, fast, and cost-effective preparation of the translation-competent cell-free extract (CFE) from budding yeast. Our protocol streamlines steps combined from different procedures published over the last three decades and incorporates cryogenic lysis of yeast cells to produce a high yield of the translationally active material. We also describe techniques for the validation and troubleshooting of the quality and translational activity of the obtained yeast CFE.

Keywords: *Saccharomyces cerevisiae*, Cell-free translation, Protein expression, Cryogenic lysis, Gel-filtration, RNA

This protocol was validated in: Sci Rep. (2021), DOI: 10.1038/s41598-021-82025-6.

Graphical Abstract:



The flow of Cell-Free Extract (CFE) preparation procedure.

Background

Cell-free systems for protein synthesis make use of specially prepared cell extracts that retain macromolecular components required for translation, including ribosomes, tRNAs, aminoacyl tRNA synthetases, protein translation factors, and, in some cases, endogenous cellular mRNAs. The cell-free reactions can be charged with an mRNA of choice supplemented with radiolabeled or otherwise modified amino acids that become incorporated into nascent polypeptide chains during translation.

Cell-free translational platforms have been developed and optimized over the years for a plethora of organisms, such as *E. coli* (Shrestha *et al.*, 2012; Kim *et al.*, 2019), *Bacillus subtilis* (Kelwick *et al.*, 2016), *Pseudomonas putida* (Wang *et al.*, 2018), *Streptomyces* strains (Li *et al.*, 2017), *Leishmania trypanosomes* (Kovtun *et al.*, 2011), fungi *Neurospora crassa* (Wu *et al.*, 2018), tobacco Bright Yellow 2 (BY-2) cells (Buntru *et al.*, 2014), insect cells (Ezure *et al.*, 2014), Chinese hamster ovary cells (CHO) (Brödel *et al.*, 2014; Thoring and Kubick, 2018), and HeLa cells (Witherell, 2001; Mikami *et al.*, 2010). For eukaryotic organisms, the most well-known and widely used cell-free translation systems have been those based on the rabbit reticulocyte (Pelham and Jackson, 1992; Olliver and Boyd, 1996) and wheat germ lysates (Harbers, 2014); both types are commercially available nowadays in several formulations. Besides generating both labeled and unlabeled proteins in a cell-free context, these reagents aided significantly in studying molecular mechanisms of protein synthesis, providing insights into individual steps of translation, translational efficiency, fidelity, and ribosome-associated protein quality control. In addition, cell-free translation systems have been instrumental in studies of protein evolution, enzyme engineering, high-throughput screens, and production of large quantities of proteins of interest for functional and structural analysis (reviewed in Carlson *et al.*, 2012; Chong, 2014; Gregorio *et al.*, 2019).

The budding yeast *Saccharomyces cerevisiae* represents an excellent source for translation-competent lysates. This unicellular eukaryotic organism combines the benefits of cost-effective microbial culture with an assortment of well-developed genetic tools: strains of interest can be easily generated with genes deleted (non-essential genes), depleted (essential genes), or overproduced. Moreover, a multitude of genetically modified yeast strains is already available from research laboratories and commercial sources. Despite many benefits, yeast-based translation extracts are not commercially available at present. Although several laboratory protocols have been published, many are too lengthy or incorporate cumbersome experimental manipulations, the rationale for which is not always clear.

Guided by the published protocols, we developed a streamlined procedure, described below, that allows efficiently generating translation-competent *S. cerevisiae* extracts. Our main goal was to improve and simplify this technology to make it more user-friendly, reliable, reproducible, and affordable for any laboratory.

Unlike the previously documented lysis procedures that utilize enzymatic cell wall digestion and spheroplasting of yeast cells (Gasior *et al.*, 1979; Tuite and Plessset, 1986) or the mechanical yeast cell lysis (Hodgman and Jewett, 2013; Hussain and Leibowitz, 1986; Schoborg *et al.*, 2014; Tarun and Sachs, 1995; Tuite and Plessset, 1986; Tuite *et al.*, 1980), we adapted a cryogenic lysis technique. This approach has several advantages over conventional lysis procedures: the translationally important components are protected from excessive degradation; precise amounts of cellular material can be taken for cryogenic grinding/milling and lysis, improving reproducibility between lysate preparations or among different strains. In addition, frozen yeast can be stored at -80°C either prior to or after cryogrinding for an extended time, creating breakpoints in the procedure. In our protocol, we adapted two consecutive centrifugation steps (at 30,000 × g and at 100,000 × g) as in procedures described by Hofbauer *et al.* (1982) and Tuite and Plessset (1986). These steps allow separation of the translationally active cellular fraction from heavy particles, lipids and polysaccharides. After a gel filtration step on Sephadex G-25 columns, we use a glycerol-containing buffer, which in our hands results in longer storage of an active lysate. Unlike many published protocols (Hodgman and Jewett, 2013; Hussain and Leibowitz, 1986; Schoborg *et al.*, 2014; Tarun and Sachs, 1995; Tuite and Plessset, 1986), we omitted treatment of the cell-free extract (CFE) with micrococcal nuclease, designed to remove the endogenous mRNA. Although the cellular mRNA might interfere with translation of the reporter, we find that for many types of experiments, this step is unnecessary, and it also tends to lead to high variability between different CFE batches.

Here, we provide a detailed protocol for preparing translationally competent lysates from budding yeast, which has been applied in our work to study unconventional translation initiation mechanisms driven by 5'-UTRs (Trainor *et al.*, 2021).

Materials and Reagents

1. PD-10 columns Sephadex G-25 (20 × 80 mm) (GE Healthcare, catalog number: 17085101); store at room temperature
2. 15 mL conical centrifuge tubes (RNase-free) (VWR, catalog number: 10026-076) or equivalent
3. 50 mL conical centrifuge tubes (RNase-free) (VWR, catalog number: 10026-078) or equivalent
4. 1.7 mL microcentrifuge tubes (RNase-free) (Denville, catalog number: C2170) or equivalent
5. PCR tubes (USA Scientific, catalog number: 1402-2900) or equivalent
6. Ultracentrifuge tubes (Beckman Coulter, model: 10.4 mL polycarbonate bottles with cap assembly (16 × 76 mm) for the Ti80 rotor; catalog number: 355603)
7. Volumetric Serological pipettes, RNase-free, for 25 mL (Denville Scientific Inc, catalog number: P7129) and for 5 ml (Denville Scientific Inc, catalog number: P7127) or equivalent
8. 25 mm Syringe filter (Corning, catalog number: 431224)
9. Rapid flow bottle top PES filter, 500 mL (Thermo Scientific, catalog number: 73520-994)
10. Sterile Pasteur pipettes (Thomas Scientific, SteriPettes, catalog number: 1215D97) or equivalent
11. BY4741 strain (Fisher Scientific; Dharmacon Inc, YEAST PARENT STRAIN BY4741, YSC1048); store in YPD/20% glycerol stock at -80°C
12. Peptone (Research Products International, catalog number: P20250); store at room temperature
13. Yeast extract (Thermo Scientific, catalog number: J23547-A1); store at room temperature
14. Dextrose (VWR, BDH Chemicals, catalog number: BDH9230); store at room temperature
15. Bacto-agar (VWR, Life Sciences, catalog number: J637); store at room temperature
16. RNase away solution (Thomas Scientific, catalog number: 1236B62); store at room temperature
17. HEPES free acid (VWR Life Sciences, catalog number: 0511); store at room temperature
18. Potassium acetate, C₂H₃O₂K (Bio Basic, catalog number: PB0438); store at room temperature
19. Magnesium acetate tetrahydrate, C₄H₆MgO₄·4H₂O (Sigma, catalog number: M5661); store at room temperature
20. Mannitol powder (VWR BDH Chemicals, catalog number: BDH9248); store at room temperature

21. Dithiothreitol (DTT) (Sigma, catalog number: D0632); store at 4°C
22. Renilla luciferase plasmid, pRL-null (Promega, catalog number: E2271); store at -20°C
23. Firefly plasmid, pGEM-luc (Promega, catalog number: E154A); store at -20°C
24. Nano luciferase plasmid, pF4Ag NanoLuc (Addgene, catalog number: 137777); store at -20°C
25. DNA clean & concentrator kit (ZYMO RESEARCH, catalog number: D4004); store at room temperature
26. mMESSAGE mMACHINE™ T7 Transcription kit (Thermo Fisher Scientific, Invitrogen, catalog number: AM1344); store at -20°C
27. Creatine kinase, rabbit muscle (BioVision, catalog number: P1301); store at -20°C
28. Creatine phosphate (VWR, catalog number: 97061-328); store at -20°C
29. GTP solution, 100 mM (Thermo Fisher Scientific, catalog number: R0461); store at -20°C
30. RiboLock RNase inhibitor, 40 U/μL (ThermoFisher Scientific, catalog number: EO0381); store at -20°C
31. *Renilla* Luciferase Assay System (Promega, catalog number: E2810); store at -20°C
32. Firefly Luciferase Assay Systems (Promega, catalog numbers: E1500, E4030, E4550) store at -20°C
33. Nano-Glo Luciferase Assay Systems (Promega, catalog numbers: N1110, N1120) store at -20°C
34. *Renilla* luciferase monoclonal antibody (Thermo Fisher Scientific, catalog number: SR07-830), store at 4°C
35. TAP polyclonal antibody (Thermo Fisher Scientific, catalog number: CAB1001), store at 4°C
36. Rpl3 monoclonal antibody (ScRPL3, Developmental Studies Hybridoma Bank, University of Iowa), store at 4°C
37. EasyTag EXPRESS³⁵S Protein Labeling mix, [³⁵S]-, 2 mCi, 11 mCi/ml (PerkinElmer, catalog number: NEG772002MC), store at 4°C
38. Trichloroacetic acid, TCA (Sigma, catalog number: T6399); store at 4°C
39. UltraPure Agarose (Invitrogen, catalog number: 16500); store at room temperature
40. Formamide (Sigma, catalog number: 47670-25ML-F); store in 1 ml aliquots at -80°C
41. SYBR gold nucleic acid gel stain (Thermo Fisher Scientific, Invitrogen, catalog number: S11494); store at -20°C
42. Optional: Hybond-N+ Nylon membrane (GE Healthcare, catalog number: NS0921); store at room temperature
43. Optional: TRI-REAGENT-LS (Molecular Research Center Inc., catalog number: TS 120); store at 4°C
44. HCl (GFS Chemicals, catalog number: 43491); store at room temperature
45. Glycerol (VWR Life Sciences, catalog number: BDH1172); store at room temperature
46. Liquid nitrogen
47. DreamTaq PCR master mix (2×) (ThermoFisher Scientific, catalog number: K1071); store at -20°C
48. GeneRuler DNA ladder mix (Thermo Fisher Scientific, catalog number: SM0333); short term storage 4°C, long term storage -20°C
49. ATP solution, 100 mM (Thermo Fisher Scientific, catalog number: R0441); store at -20°C
50. 1 mM solution of 20 Essential amino acids, complete (Promega, catalog number: L4461); store at -20°C
51. 1 mM solution of Essential amino acids minus Methionine and Cysteine (Promega, catalog number: L5511); store at -20°C
52. Formaldehyde solution, 37% (GFS Chemicals, catalog number: 40301); store at room temperature
53. EDTA, 0.5 M sterile solution, RNase-free (VWR Life Sciences, catalog number: E522-100ML); store at room temperature
54. Yeast medium (see Recipes)
 - YPD medium
 - YPD-agar plates
55. Stock solutions (see Recipes)
 - 1 M HEPES-KOH, pH 7.6
 - 2 M KOAc
 - 300 mM MgOAc
 - 100 mM MgOAc
 - 1 M DTT
 - 50% glycerol
56. Working solutions (see Recipes)
 - Buffer A

Buffer A/glycerol
Buffer A/mannitol
Buffer A/mannitol/DTT
Buffer A/glycerol/DTT
Creatine kinase dilution buffer
Creatine kinase solution
10× Energy mix
FAE solution

Equipment

1. -80°C freezer
2. -20°C freezer
3. Lab water purification system (PureLab, model: Elga water polisher system) or equivalent
4. pH meter (Sartotius, model: PB-11-P11.1) or equivalent
5. Optional: Laminar Flow Hood (NuAir, NU-201-430)
6. Microbiological incubator (Binder World, Series ED) or equivalent
7. Incubator shaker (Eppendorf, model: New Brunswick Innova® 43) or equivalent temperature-controlled shaker that can fit a 4 L flask
8. Preparative centrifuge (Beckman Coulter, model: J2-MI) or equivalent
9. Beckman JLA-10.500 rotor (Beckman Coulter) or equivalent
10. Preparative ultracentrifuge (Beckman Coulter, model: Le-80)
11. Beckman ultracentrifuge rotor (Beckman Coulter, model: Ti80)
12. Eppendorf centrifuge with the A-4-62 rotor, refrigerated (Eppendorf, model: 5810R)
13. Benchtop centrifuge (Eppendorf, model: 5420)
14. Freezer/Mill® (SPEX SamplePrep, model: 6700) or equivalent Cryogenic grinder
15. Freezer/Mill® accessories: small grinding vial set (SPEX SamplePrep, catalog number: 6751)
16. Toplanding balance (Sartorius, catalog number: CP3202P) or equivalent
17. Pipetman (Gilson, P20, P200, and P1000) or equivalent
18. Vortexer (any model)
19. Spectrophotometer (Eppendorf, catalog number: 6131-26951) or equivalent
20. 500 mL Erlenmeyer flask
21. 4 L Erlenmeyer flask
22. PPCO centrifuge bottles with sealing closure, 500 ml (Thomas Scientific, catalog number: 2625H84) or equivalent
23. Thermocycler (Eppendorf, model: Mastercycler®) or equivalent
24. Dry bath incubator (Eppendorf, model: Thermomixer R)
25. Luminometer (Promega; model: Glomax 20/20) or equivalent
26. Liquid scintillation counter (Beckman LS System, model: LS6000TA) or equivalent
27. Power supply (Bio-Rad, model: PowerPac Universal Power Supply, catalog number: 1645070) or equivalent
28. Agarose gel electrophoresis equipment (Mansour and Pestov, 2013)
29. Gel rocker platform (Thomas Scientific, BioRocker™ 3D Mini Rockers, catalog number: 1155J96) or equivalent
30. Typhoon imager (GE, model: Typhoon 5 Biomolecular Imager), or any gel-imaging system (for example, Cole-Parmer, model: DelLogic 100 imaging system, or equivalent)
31. Protein gel electrophoresis equipment (Bio-Rad, model: Mini-PROTEAN tetra Vertical Electrophoresis Cell, catalog number: 1658004) or equivalent
32. Optional: polyacrylamide gel electrophoresis equipment (Hoefer Inc, model: SE260 Mighty Small II Delux Mini Vertical electrophoresis Unit)
33. Optional: Transfer Cell (Bio-Rad, Trans-Blot® SD Semi-Dry Transfer Cell, catalog number: 1703940) or equivalent

34. Optional: Hybridization oven (UVP, model: HB-1000 Hybridizer) or equivalent

Procedure

The preparation of a translationally active yeast CFE can be divided into three stages: (1) Growth, harvesting, and cryogenic disruption of yeast cells; (2) Isolation of a translationally active fraction from the total cell lysate; and (3) Validation of the CFE activity in translation. The procedure described below is for a 1 L yeast culture of BY4741 cells. This is the minimal volume we recommend for a CFE preparation. The protocol can also be scaled up if desired.

A. Growth, harvesting, and cryogenic disruption of yeast cells

The objective of this part of the procedure is to prepare cryomilled yeast cell powder from which the CFE will be derived.

1. Growing yeast on a plate (2-4 days in advance)
Streak the yeast strain of interest on a YPD-agar plate to achieve single-colony growth. Incubate the plate in an incubator at the temperature optimal for the strain until colonies are ~1-1.5 mm in diameter. We usually use the BY4741 strain and its derivatives for this procedure. Depending on the strain's genotype, colonies may take between 2 to 4 days to grow at 30°C.
2. General preparation and starting a yeast culture (Day 1, ~2-3 h)
 - a. Make sure that yeast media and all solutions required for the procedure have been prepared and stored at the appropriate temperature.
 - b. Autoclave 2-3 L of deionized H₂O and store it at 4°C (referred hereafter to as "cold diH₂O").
 - c. Autoclave large centrifuge bottles (400 mL or analogous). Place the autoclaved centrifuge bottles, a bag of 50 mL, a bag of 15 ml conical centrifuge tubes, and a bag of 1.7 mL microcentrifuge tubes at -20°C.
 - d. Inoculate one large yeast colony into 100 mL of YPD medium in a sterile 500 mL flask. Place the flask into an orbital shaker and grow the culture for 14-20 h at 30°C with moderate shaking (~160 rpm).
3. Growing and harvesting yeast cells (Day 2, ~6-7 h)
 - a. Inoculate the overnight culture started at Step A2d into 1 L of fresh YPD in a 4 L flask to obtain an OD_{λ=600nm} ~0.8. Incubate this culture at 30°C for 5-6 h with constant shaking until OD_{λ=600nm} ~2.5. In our experience, any deviation from these parameters may significantly lower either the CFE yield or its activity in translation reactions. For slow-growing or non-BY4741 strains, optimization of the dilution factor may be required. Avoid prolonged incubation of the day culture, which can deplete dextrose from the medium and alter cell metabolism.
 - b. While the culture is growing, cool down to 4°C all rotors and centrifuges used at Steps A3c-A3e below (e.g., a JLA-10.500 preparative rotor for the large centrifuge bottles with a Beckman J2-MI centrifuge, and an A-4-62 rotor for 50 mL tubes used with an Eppendorf 5810R centrifuge).
 - c. When the cell culture density reaches OD_{λ=600nm} ~2.5, pour the cell culture into the prechilled large centrifuge bottles. Harvest cells at 3,700 × g for 6 min at 4°C (5,500 rpm in a Beckman JLA-10.500 rotor).
 - d. Discard the supernatant, resuspend the cell pellets in a total volume of 250 mL of cold diH₂O. Pellet diH₂O-washed cells at 3,700 × g for 6 min at 4°C (5,500 rpm in a Beckman JLA-10.500 rotor). To resuspend cell pellets, we use a volumetric 25 mL plastic pipet and gently swirl centrifuge bottles periodically until a homogenous cell suspension is formed. Do not vortex the cell pellets. We typically divide cell suspensions obtained at this step between two centrifuge bottles, which makes it easier to resuspend cell pellets at the next step.
 - e. Discard the supernatant, resuspend the cell pellets in a total volume of 100 mL of cold diH₂O. Working on ice, transfer the cell suspension into two cold, sterile 50 mL centrifuge tubes. Pellet

cells at $3,220 \times g$ for 5 min at 4°C (4,000 rpm in an Eppendorf 5810R centrifuge with the A-4-62 rotor).

4. Preparation of the frozen cell/buffer beads (Day 2, ~2 h)

Keep centrifuge tubes containing yeast cells on ice during all steps. Use appropriate safety precautions and wear eye protection when working with liquid nitrogen.

- a. Wash cell pellets once with cold buffer A/mannitol and once with cold buffer A/mannitol/DTT (25 mL per tube; centrifuge cells as in Step A3e above). Combine cell suspensions in one tube during the last wash.
- b. Use a Pasteur pipet attached to a vacuum flask to aspirate off the excess buffer from the tube. Determine the weight of the yeast cell pellet. Use an empty 50 mL centrifuge tube to tare the balance. Normally, we obtain ~5 g of the wet cell material from a 1 L yeast culture grown to a final $\text{OD}_{\lambda=600\text{nm}} \sim 2.5$.
- c. Resuspend the yeast pellet in buffer A/mannitol/DTT at a 2:3 volume (mL):pellet weight (g) ratio. For example, for 5 g of yeast cells, use 3.3 mL of the buffer. The cell suspension will be very thick. Make sure you keep the cell suspension on ice.
- d. Pour ~25 mL of liquid nitrogen into clean 50 mL "collector" tubes secured in a plastic rack. Place the rack inside an ice bucket or a clean styrofoam container partially filled with liquid nitrogen (as shown in Figure 1A). Wait for ~10 min to ensure a thorough cooldown of the entire setup.
- e. Collect the cell suspension from Step A4c above into a 5 mL volumetric plastic pipet. Keeping the pipet tip ~5-6 cm above the liquid nitrogen's level to avoid tip freezing (Figure 1A), eject the cell suspension dropwise into a liquid nitrogen-filled collector tube. To form individual cell/buffer beads, allow each drop to freeze before adding the next one; adding drops too quickly will cause clump formation, which complicates loading the beads into a grinding vial at a later step of the procedure. Add more liquid nitrogen into the collector tube if needed.

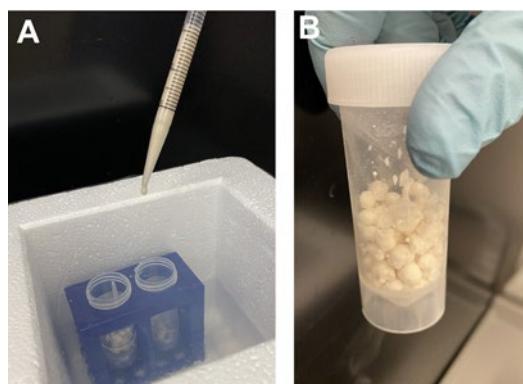


Figure 1. Illustration of the frozen cell/buffer beads preparation for cryolysis.

To ensure well-separated beads, slowly eject cell suspension with an interval of 3-5 s between drops. Using two collector tubes and switching between them for each drop (as shown on panel A) can speed up the process while allowing the drops to freeze properly. Cell/buffer beads should then be combined in a single tube for storage, as shown in panel B.

- f. When the last drop is frozen, carefully discard excess liquid nitrogen from the collector tubes. Cap the tubes loosely to allow outgassing and place the frozen cell/buffer beads (Figure 1B) at -80°C for storage. The frozen cell/buffer beads can be used immediately for the next steps. For a break in the procedure, beads can be kept at -80°C from overnight to several weeks. Typically, we obtain ~7-8 g of the cell/buffer beads from 5 g of the wet cell pellet Step A4b.

5. Cryogenic grinding of yeast cells (Day 2 or Day 3, ~2 h)

In this section, we provide the instructions and illustration for the SPEX SamplePrep Freezer/Mill® Model 6700. Other, more advanced Freezer/Mill models are available commercially. Use appropriate

safety precautions and wear eye protection when working with liquid nitrogen.

- a. Fill the cryogenic mill's chamber with liquid nitrogen following the manufacturer's instructions.
- b. Place grinding vials with the small end plugs attached and impactor rods placed inside (see Figure 2A, parts a, b, and c) vertically into a styrofoam container partially filled with liquid nitrogen (Figure 2B). Be sure to only submerge the vials halfway in liquid nitrogen, as excessive cooling of their tops will shrink them too much and prevent locking of the large end plugs (Figure 2A, part d) at a later step. Allow the chamber and vials to cool down for 15-30 min. Normally, for 7-8 g of the cell/buffer beads obtained in Step A4f, we use two small polycarbonate grinding vials fitted with individual stainless-steel impactor rods.
- c. Blank the weight of a grinding vial (with a rod inside) on a balance. Shake cell/buffer beads inside the vial to get 4-4.5 g of the beads per vial (Figure 2D). Place the vial filled with beads back into the liquid nitrogen in a vertical position for 2-3 min (Figure 2C). Work fast to avoid thawing the cell/buffer beads.
- d. Close the vial with a large end plug (Figure 2E) and position the locked vial horizontally inside the liquid nitrogen container so that the vial is completely submerged (Figure 2F).
- e. Repeat Steps A5c-A5d with the remaining vial(s).

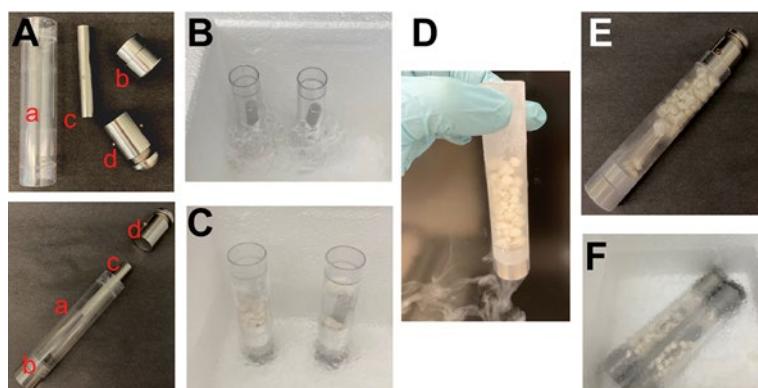


Figure 2. Preparation of grinding vials for cryomilling.

A. Parts of a grinding vial: polycarbonate vial (a); small (bottom) end plug (b), impactor rod (c), large (top) end plug (d). Parts b and d may look slightly different depending on cryomill model. B. Prechilling grinding vials in liquid nitrogen. C, D. Grinding vials with cell/buffer beads inside. E. A vial closed with the top end plug. F. Locked grinding vials loaded with beads, ready for cryomilling.

- f. Place a grinding vial loaded with beads inside the cryomill chamber (Figure 3A), lock the chamber, and wait 10 min for the system's complete cooling. Grind the cells using the maximum speed of the SPEX Freezer/Mill® 6700 and the following settings: 1 min grinding, 1 min rest, for a total of 8-10 cycles. Add liquid nitrogen to the chamber if needed, as multiple cycles could lead to partial nitrogen evaporation. After the last cycle, wait 10 min before transferring the vial to a -80°C freezer. We recommend storing powdered cells inside unopened grinding vials (Figure 3B) at -80°C for at least 30 min (overnight is also an option) to allow for outgassing of any residual nitrogen. For long-term storage, the powder can be stored at -80°C for 2-3 months.

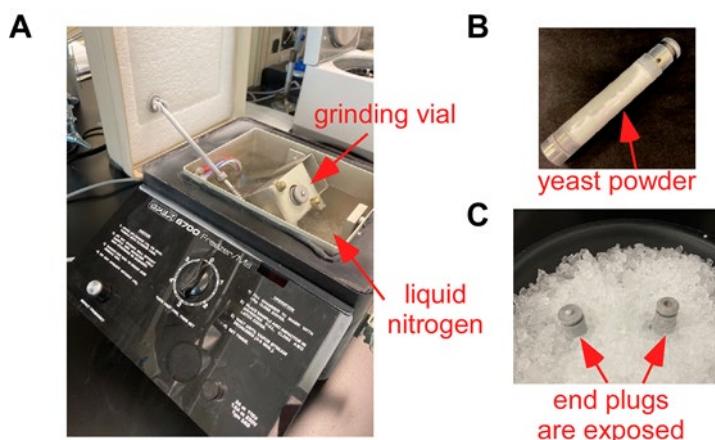


Figure 3. Cryogenic cell grinding.

A. Cryogenic mill filled with liquid nitrogen and a grinding vial loaded into the device. B. Powdered yeast cells inside a grinding vial. C. Correct placement of grinding vials containing yeast powder in an ice bucket, making the top ends exposed to the room-temperature air.

B. Isolation of a translationally active fraction from the cell lysate

The main objective of this part of the procedure is to separate the translationally active fraction of the cell lysate from other lysate components that are unnecessary, and potentially inhibitory, for a translation reaction. Desalting on Sephadex G-25 columns removes low molecular weight compounds from the CFE, including endogenous amino acids and nucleotides. All steps described in (B) must be performed on the same day.

1. Cell lysis (Day 3, ~6 h)
 - a. Prechill a fixed-angle rotor (*e.g.*, a Beckman Ti80 rotor) and an ultracentrifuge to 4°C.
 - b. Transfer grinding vials containing cryomilled yeast powder (Figure 3B) from -80°C to an ice bucket. Position the vials vertically so that the top end plugs are exposed to the room-temperature air (Figure 3C). Place two 50 ml conical tubes and four capped small polycarbonate ultracentrifuge tubes into the ice bucket.
 - c. After keeping the grinding vials on ice for ~5 min, remove the top end plugs and shake the yeast powder from the vials into a prechilled 50 ml tube. Keeping the vials on ice for too long may cause thawing of the yeast powder, making it resemble a paste. In that case, use a clean RNase-free spatula to transfer the material into the 50 mL tube.
 - d. Add 2-3 mL of buffer A/mannitol/DTT to the 50 mL tube.
 - e. Use 3-4 mL of buffer A/mannitol/DTT to collect the residual amounts of the cell material left on the walls of the grinding vial and the spatula. Add the recovered cell material to the 50 mL tube containing the bulk of the cryomilled yeast powder (B1c).
 - f. Pipet the mixture up and down to obtain a homogenous slurry of the broken yeast cells and the buffer. The lysate will be very thick at this point. Add additional buffer A/mannitol/DTT to the final volume of 9 mL and pipet to mix. Transfer the lysate into the prechilled small ultracentrifuge tube. Rinse the 50 mL tube with 1 mL of the remaining buffer to collect the leftover material and transfer it into the ultracentrifuge tube. The total volume of the lysate must be 10 mL. Close the ultracentrifuge tube with a cap.
 - g. If the lysate is not completely thawed and slushy, incubate the ultracentrifuge tubes containing the yeast suspension on ice for 5-15 min.
 - h. Place the ultracentrifuge tubes into a rotor and centrifuge them at 30,000 × g (17,000 rpm in Beckman Ti80 rotor) for 15 min at 4°C.
 - i. Carefully remove the tubes from the centrifuge rotor and place them on ice vertically. You will see three fractions, as shown in Figure 4. Transfer 6 ml of the middle fraction ("S30") into a new

prechilled small polycarbonate ultracentrifuge tube while avoiding any lipid layer at the top. We successfully used a Pipetman P1000 set at 1 mL to collect the middle fraction. However, to avoid repeated crossing through the top layer, one can also use a 10 mL syringe with an attached needle. Be careful not to aspirate the top or bottom fractions. Keep tubes on ice and work fast to minimize the lysate exposure to room temperature.

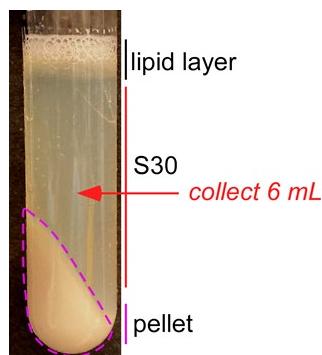


Figure 4. Formation of three distinct fractions after the $30,000 \times g$ centrifugation.

Top: milky-white layer of lipids. Middle (marked as S30): the yellowish soluble fraction that contains the translationally active material. Bottom: large pellet containing unbroken cells, cellular debris, and DNA.

- j. Place the ultracentrifuge tubes into the rotor and centrifuge them at $100,000 \times g$ (30,000 rpm in a Beckman Ti80 rotor) for 35 min at 4°C .
- k. While the tubes are spinning, it is a good time to start preparing gel-filtration columns used for lysate desalting (see Step B2a below).
- l. When centrifugation is complete (B1j), carefully remove the ultracentrifuge tubes from the rotor and place them on ice vertically for 5-10 min (see Step B1m). Avoid disturbing the tube contents. Carefully collect 2.5 mL of the S100 fraction as depicted in Figure 5 (also, see Step B1m) and transfer it into a prechilled 15 mL conical tube.

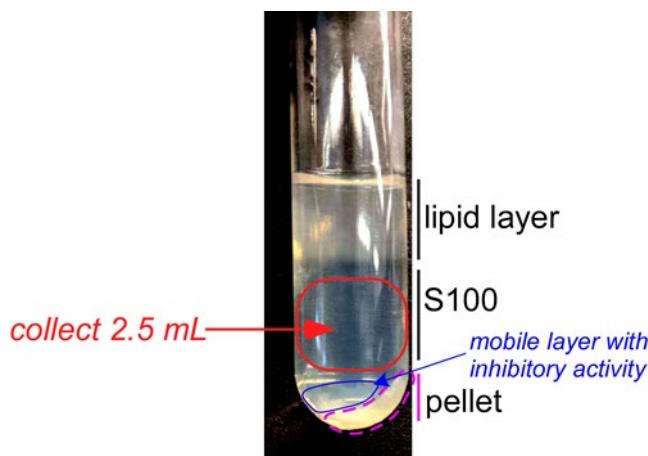


Figure 5. Formation of four distinct layers in yeast cell lysate following the $100,000 \times g$ centrifugation.

Top: Large yellowish layer containing lipids. Middle (marked as S100): translucent layer that is the desired polysome-free cell lysate fraction. Bottom: A largely immobile pellet composed of heavy cellular components, such as the ER and polysomes (traced in pink), and a mobile yellowish fraction (traced in blue).

- m. Keeping the tubes on ice for 5-10 min helps to better visualize the four layers. To collect the intermediate S100 fraction, a Pasteur pipet or a syringe with an attached needle can be used. The collection of S100 while avoiding the upper or bottom layers is a challenging step as these layers are mobile and easily intermix with the S100 fraction. Be especially careful not to contaminate S100 with the mobile pellet fraction (traced in blue in Figure 5).
2. Lysate desalting and concentration (Day 3, 45-60 min for column equilibration + ~1 h for desalting)
 - a. Remove caps from the PD-10 Sephadex G-25 columns, then cut off the bottom tips at a 45° angle. Allow the storage buffer to run out by gravity flow. Equilibrate each column with 5 × 5 ml of cold buffer A/glycerol/DTT.
 - b. During column equilibration, label ten 1.7 mL microcentrifuge tubes for collecting fractions (1-10), plus two extra tubes for the flowthrough. Place the tubes on ice.
 - c. Transfer the equilibrated columns, microcentrifuge tubes, and lysates from Step B11 into a cold (4°C) room. Make sure that the columns have no buffer left at the top. Place the microcentrifuge tubes in a rack, arrange them so that the first flowthrough tube is directly underneath the outlet of a PD-10 Sephadex G-25 column clamped to a laboratory stand.
 - d. Carefully apply 2.5 mL of lysate from Step B11 to the top of the column and allow the sample to completely enter the column bed. Collect the flowthrough using two microcentrifuge tubes. For elution, pipet 5 ml of buffer A/glycerol/DTT onto the column bed and begin collecting fractions into 1.7 mL microcentrifuge tubes labeled 1-10. Collect ~0.5 mL per tube. Cap each tube. Gently vortex or pipet up and down the tube content. Place all the tubes on ice.
 - e. Measure the RNA concentration in each tube: dilute a portion of each fraction with H₂O 1:100 and measure the absorbance at $\lambda = 260$ nm using a spectrophotometer. To calculate RNA concentration (in $\mu\text{g}/\mu\text{l}$), multiply the absorbance by the RNA extinction coefficient ($\epsilon_{\text{RNA}} = 40$) and by the dilution factor (100). Plot the RNA concentration in each fraction on a graph, as shown in Figure 6.

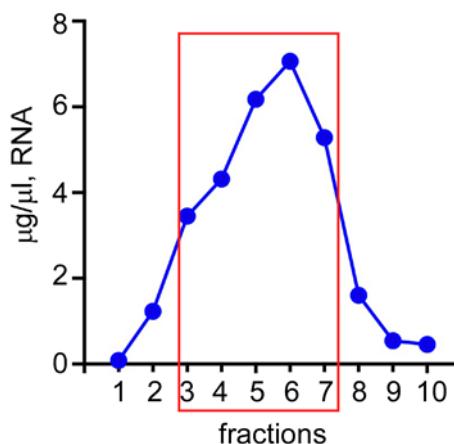


Figure 6. Graphical representation of the total RNA distribution in the G-25 column elution fractions.

RNA concentration in each fraction was determined spectrophotometrically: a portion of each fraction was diluted with H₂O (1:100), and the absorbance was measured at $\lambda = 260$ nm. RNA concentration was calculated ($\epsilon_{\text{RNA}} = 40$), converted to $\mu\text{g}/\mu\text{l}$, and plotted on a graph. We collect fractions with at least 60-75% RNA compared to the highest containing fraction. Typically, these are fractions 3-7 (boxed in red).

- f. Combine fractions with the highest RNA concentration in one 15 mL tube and thoroughly mix the content by gentle vortexing or pipetting it up and down. Working on ice, aliquot the resulting CFE into prechilled 1.7 mL microcentrifuge tubes, 100 μl per tube. Snap-freeze the CFE aliquots in liquid nitrogen and place the tubes at -80°C for storage. An example of the yeast CFE preparation

procedure is in Table 1.

Table 1. An example of the yeast Cell-Free Extract (CFE) preparation procedure

Starter culture	Day culture	Growth	Harvest culture	Cell pellet, weight	Cells/buffer beads, weight	CFE, RNA conc.	CFE, protein conc.	Number of reactions
70 mL, OD _{λ=600nm} ~12	1 L, OD _{λ=600nm} ~0.8	5 h, 30°C, shaking	1 L OD _{λ=600nm} ~2.5	5.3 g	7.6 g	4.5 μg/μL	12.5 μg/μL	~660

Using the described procedure, from 1 L of yeast culture grown to OD_{λ=600nm} ~2.5 (column 4), we generate 7-8 g of cell/buffer beads (column 6). The cryogenic lysis of the cell/buffer bead material is performed in two separate vials, resulting in two 2.5 mL aliquots of S100, followed by the two desalting/concentrating steps using two individual PD-10 Sephadex G-25 columns. The volume of the generated eluate from one column with the highest RNA concentration is 2.5 mL. By combining eluates from two columns, we obtain 5 mL of the CFE, which is enough for ~660 standard-size reactions.

C. Validation of the CFE activity in translation

To determine the translational activity of a newly prepared CFE, set up a translation reaction with an mRNA reporter (section 1 below) or, alternatively, use [³⁵S]-methionine/cysteine labeling of translation products derived from the endogenous mRNA present in the CFE (section 2). An optional analysis of RNA integrity (section 3) may be useful for troubleshooting the extract preparation procedure.

1. Validation of the translational activity of CFE using luciferase reporters

a. Generation of the reporter mRNA

The protocol below is designed for the Renilla luciferase construct, as used in Trainor *et al.* (2021). Refer to Table 2 for details on using other luciferases, such as Firefly and NanoLuc®, as the protein reporters in CFE-based translation reactions.

Table 2. Synthesis and detection of Firefly and NanoLuc® luciferase-based protein reporters

Luciferase	Template	Forward primer	Reverse primer	Detection kit	
Firefly	pGEM-luc Promega (E154A)	5'-ATCCGTAAATACGCTCACTAT AGGGAGCTTCACACTAAATTAA ATATGGAAGACGCCAA AACATAAAGAAA-3'	5'-TTACAATTT GGACTTCCGCC CTTC-3'	Luciferase System, Promega (E1500, E4030, E4550)	Assay
Nano	pF4Ag NanoLuc, Addgene (137777)	5'-ATCCGTAAATACGACTCACTA TAGGGTAACCGAGCTCGGACT ATGGTCTTCACACTCGAAGATT TCG-3'	5'-TTACGCCAG AATGCGTTCGCA CA-3'	Nano-Glo Luciferase Assay system, Promega (N1110, N1120)	

The T7 promoter sequence is marked in blue; the CYC1 5'-UTR translational enhancer used to increase translation of the Firefly luciferase is marked in red.

- Amplify the *Renilla* luciferase gene by PCR using the following primers: the forward primer (5'-ATCCGTAAATACGACTCACTATAGGGTAACCGAGCTCGGACT**ATG**
ACTTCGAAAGTTATGATCCAGAA), the reverse primer (5'-TCACGACATTG
TTCATTTTGAGAACTCG-3'). The forward primer contains a T7 polymerase promoter

sequence (bolded); the start ATG codon is highlighted in red.

Set up a PCR reaction in a total volume of 50 μ L by mixing 25 μ L of the DreamTaq PCR master mix (2 \times), 1 μ L of 20 μ M forward primer, 1 μ L of 20 μ M reverse primer, 1 μ L of the template plasmid (10 ng/ μ L), and 22 μ L of H₂O. A *Renilla* luciferase gene-containing plasmid can be purchased from Promega (see Materials). Amplify DNA using the following PCR program: 27-30 cycles at 94°C for 30 s (denaturation), 54°C for 30 s (annealing), and 72°C for 1 min (polymerization).

- ii. Upon completion of the PCR, check 5 μ L of the reaction on a 1% agarose gel. The *Renilla* luciferase gene is 940 bp. Use a molecular weight marker to determine the size of the PCR product. If you detect additional bands, repeat the PCR reaction with an increased primer annealing temperature to achieve a single 940 bp band.
 - iii. Purify the PCR product using the DNA clean & concentrator kit spin columns following the manufacturer's protocol. Elute the purified PCR product from a column with 16 μ L of H₂O. Measure DNA concentration using a spectrophotometer.
 - iv. Generate m7G-capped mRNA using 1 μ g of the purified PCR DNA (from Step C1a.iii) in a transcription/capping reaction with an mMESSAGE mMACHINE™ T7 transcription kit. Incubate the reaction at 37°C for 2 h. Follow the kit protocol for purifying the transcription product, including DNase treatment for 15 min at 37°C and RNA precipitation with LiCl. Finish with washing the precipitated RNA with 80% EtOH and air-drying the RNA pellet.
 - v. Resuspend the RNA pellet in 30-40 μ L of RNase-free H₂O by repeated pipetting. Keep the RNA on ice. Measure RNA concentration using a spectrophotometer as described in Step B2e. Aliquot 4-5 μ L of the generated RNA into PCR tubes and freeze these aliquots at -80°C. For consistency, we do not recommend freeze-thaw cycles of the reporter mRNA.
- b. Perform the *in vitro* translation reactions
- This protocol is based on Wu and Sachs (2014) with some minor modifications. To ensure reproducibility and for statistical analysis, assemble each reaction in triplicate.
- i. Prepare the 2.5 \times master mix (required volume: 80 μ L for 12 reactions) by assembling its components in the order indicated in Table 3. Keep the master mix on ice.

Table 3. Preparation of the 2.5 \times master mix for translation reactions

Reagent	Volume
1 H ₂ O	45.8 μ L
2 2 M KOAc	5 μ L
3 0.1 M MgOAc	4 μ L
4 10 \times Energy mix (see Recipes)	20 μ L
5 1 mM amino acids	2 μ L
6 RiboLock (40 units/ μ L)	2 μ L
7 Creatine kinase (10 units/ μ L)	1.2 μ L

- ii. Thaw an mRNA aliquot on ice, adjust the concentration to 200-300 ng/ μ L using RNase-free H₂O. Keep the diluted mRNA on ice.
 - iii. Thaw an aliquot of the CFE by warming the tube in your hand and periodically tapping it gently to mix the content. As soon as the CFE is thawed, place the tube on ice.
 - iv. Set up the desired number of translation reactions by first adding 7.5 μ L of CFE to 1.7 mL microcentrifuge tubes. Add 6 μ L of the 2.5 \times master mix to each tube. Add 1.5 μ L (300-450 ng) of the mRNA into each tube. Reactions can be assembled on a bench at room temperature. The total volume of each reaction is 15 μ L. Mix the reaction components by gently tapping the tubes and centrifuge the tubes in a tabletop centrifuge for 10 s. Place the tubes into a dry bath incubator set at 21°C. Incubate the reactions for 1-2 h.
- c. Analyze the translation reaction products by a luciferase assay and/or western blotting.

- i. For a *Renilla* luciferase assay, use reagents from a *Renilla* Luciferase Assay System kit (Promega). Follow the protocol provided with the kit.
 - ii. To visualize proteins generated in the translation reaction on a gel, perform quantitative western blotting with fluorescent secondary antibodies, for example, as detailed in Ghosh and Shcherbik (2020), or by using HRP-fused secondary antibodies and an ECL detection system (Trainor *et al.*, 2021). The *Renilla* luciferase-specific antibodies are commercially available (see Materials).
2. Labeling of the nascent polypeptides with [³⁵S]-Met/Cys as a readout of the CFE translational activity
Use appropriate safety precautions when working with radioactive materials.
 - a. Prepare the 2× labeling master mix (100 µL for 12 reactions) by assembling its components in the order indicated in Table 4. Keep the master mix on ice.

Table 4. Preparation of the 2× labeling master mix for the labeling of nascent proteins with [³⁵S]-Met/Cys

Reagent	Volume
1 H ₂ O	56.8 µL
2 2 M KOAc	5 µL
3 0.1 M MgOAc	4 µL
4 10× Energy mix (see Recipes)	20 µL
5 1 mM amino acids (-Met, Cys)	2 µL
6 RiboLock (40 units/µL)	2 µL
7 Creatine kinase (10 units/µL)	1.2 µL
8 EasyTag EXPRESS ³⁵ S Protein Labeling mix, [³⁵ S], 11 mCi/mL	9 µL

- b. Thaw an aliquot of the CFE by warming the tube in your hand and gently tapping the tube to mix the content. As soon as the CFE is thawed, place the tube on ice.
 - c. Set up the translation reactions by first adding the CFE to 1.7 mL microcentrifuge tubes, 7.5 µL per tube. Next, add 7.5 µL of the 2× labeling master mix to each tube. The total volume of each reaction is 15 µL. Mix the reactions by gently tapping the tubes and centrifuge the tubes in a tabletop centrifuge for 10 s. Place the tubes into a dry bath incubator set at 21°C. Incubate the reactions for 1-2 h.
 - d. Precipitate [³⁵S]-labeled polypeptides with TCA. For example, follow the protocol described in <https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/rabbit-reticulocyte-lysate-system-protocol.pdf>.
 - e. Measure the activity of the [³⁵S]-Met/Cys]-labeled polypeptides by scintillation counting.
3. Analysis of RNA integrity

If a newly prepared CFE batch has a low translational activity, we recommend examining both the CFE and the generated mRNA reporter by gel analysis and (optionally) Northern hybridizations. This will address the potential RNase contamination of the reagents and equipment used in the procedure, which may lead to degradation of the translation reaction components.

 - a. Analysis of rRNA and tRNA integrity
 - i. Thaw one tube of the frozen CFE and measure its RNA concentration. For gel analysis, take an aliquot of the CFE and dilute it with RNase-free water to 2.5 µg/µL RNA. Mix 1 µL of this diluted CFE with 4 µL of the FAE solution (see Recipes) and heat the sample at 70°C for 5 min.
 - Be advised that the CFE obtained through the protocol described above contains a high amount of rRNA and tRNA. Dilute the CFE with H₂O 100-fold before measuring RNA concentration, as described in (B2.e). After heating the sample with FAE (Shedlovskiy *et al.*, 2017), it can be directly analyzed by the denaturing gel electrophoresis.
 - ii. Analyze large molecular weight RNA species (25S and 18S rRNAs) by electrophoresis on

- formaldehyde-agarose gels as described in Mansour and Pestov (2013). To separate small molecular weight RNA species (tRNAs, 5S and 5.8S rRNAs), use polyacrylamide denaturing gels as described in Sheherbik *et al.* (2016).
- iii. Dilute the SYBR Gold dye stock 1:10,000 (10 µL of SYBR Gold in 100 mL of H₂O). Pour this solution into a clean plastic tray and stain the gel by incubating it for 20-30 min with slow rocking at room temperature. Visualize SYBR Gold-stained RNA bands using a suitable gel-imaging system or a laser fluorescence scanner.
 - iv. (Optional) Transfer RNA from the gel to a nylon membrane and hybridize the blot with probes specific for different yeast rRNAs and tRNAs (probe sequences are provided in Table 5). Follow the hybridization protocol described in Pestov *et al.* (2008). We recommend first to use probes for 25S and 18S rRNAs, the large rRNA species whose degradation in lysates can be easily detected.

Table 5. Sequences of probes used to detect yeast rRNAs and tRNAs

Probe	Sequence
25S rRNA	5'-TCCTACCTGATTGAGGTCAAAC-3'
18S rRNA	5'-AGAATTCACCTCTGACAATTG-3'
5.8S rRNA	5'-AAATGACGCTCAAACAGGGCATG-3'
5S rRNA	5'-TAACTACAGTTGATCGGACGG-3'
tRNA^{Glu}	5'-TGGCTCCGATACTGGGGAGTCG-3'
tRNA^{Val}	5'-TGGTGATTCGCCAGGA-3'
tRNA^{Ala}	5'-GGTGGACGAGTCCCGGAATCG-3'

- b. Examine the quality of m7G-capped mRNA prior to and after the translation reaction
If a low translational activity is detected in a CFE using a protein reporter, there is a possibility that the reporter mRNA might have a secondary structure resulting in abortive synthesis by T7 polymerase. Another possibility is that mRNA generated *in vitro* might be degraded either during the synthesis or during translation reaction. To test these possibilities, proceed with the following steps:
- i. After synthesizing the mRNA reporter (C1.a), analyze 2 µg of the generated RNA by agarose gel electrophoresis followed by SYBR Gold staining, as described in Steps C3a.iii. The detection of a single band would indicate that the mRNA is intact. If you see multiple bands, repeat mRNA synthesis using the mMESSAGE mMACHINE™ T7 Transcription kit; however, heat the PCR DNA product before adding into the reaction at 65°C for 5 min followed by an immediate cooldown on the ice for 2 min. If this does not improve the quality of the generated mRNA, change all the solutions to rule out the possibility of RNase contamination. Follow other troubleshooting steps described in the mMESSAGE mMACHINE™ T7 Transcription kit manual.
 - ii. After the completion of the translation reaction (C1.b), extract RNA from the reaction using TRI-REAGENT-LS according to the manufacturer's protocol. Resuspend the RNA pellet in 50 µl of FAE (Shedlovskiy *et al.*, 2017) and analyze 2.5 µg of RNA by northern hybridization using a probe specific for the mRNA reporter. Detection of a single band indicates that the mRNA is stable in the CFE-based translation reaction. The appearance of extensive degradation products may indicate the RNase contamination of reagents, in which case we recommend changing the solutions and reviewing the laboratory setup to ensure an RNase-free environment.

Data analysis

1. If the researcher chooses to use a luciferase reporter to assess the activity of the CFE, the amount of the generated products can be quickly estimated using a commercially available luciferase assay. This is a simple, quantitative, and reliable method to verify the translational competency of a newly prepared CFE batch. An example of a luciferase-based assay is shown in Figure 7.

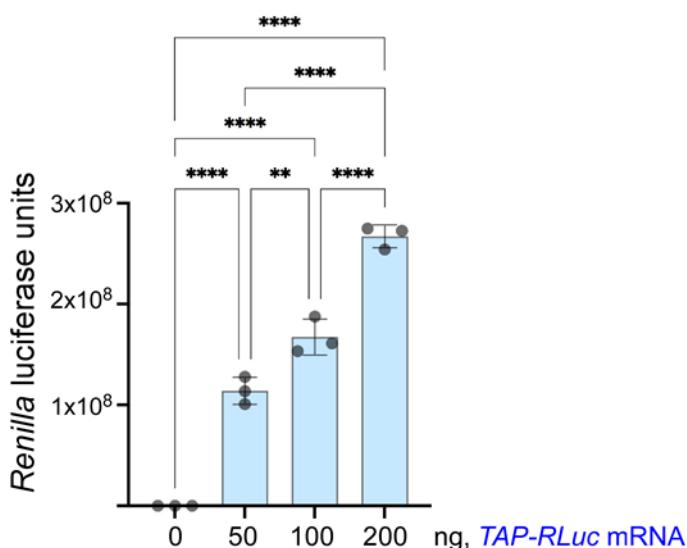


Figure 7. Detection of the TAP-RLuc reporter synthesis in a yeast CFE-based reaction.

The indicated amounts of the TAP-RLuc mRNA reporter (Trainor *et al.*, 2021) were added to CFE-based translation reactions set in triplicate. The reactions were incubated at 21°C for 2 h, and the products were analyzed using a commercial *Renilla* Luciferase Assay System (Promega). Luminescence was measured for 10 s after the addition of the detection reagent. Bars show mean values; error bars represent SEM. One-way ANOVA tests were used to compare the samples. **, $P < 0.01$; ****, $P < 0.0001$.

2. An alternative way to estimate protein amounts synthesized from a reporter in a CFE-based reaction is to separate the translation products on a gel followed by immunoblotting with the appropriately selected antibodies. In our hands, a strong signal for a TAP-fused protein reporter is evident after a 30-min translation reaction performed with an active CFE (Figure 8). This method can be used with any protein reporter for which antibodies are available for immunodetection.

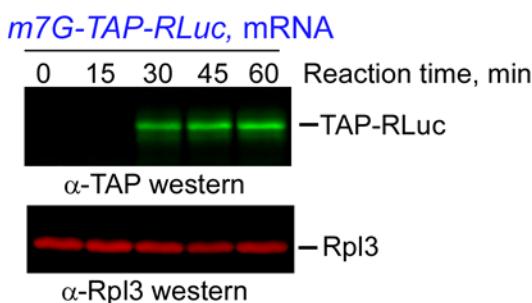


Figure 8. Immunoblot detection of a TAP-fused reporter translated in a yeast CFE.

Aliquots of 2 µL were taken from a translation reaction at the indicated times, mixed with 2 µL of 2× SDS-PAGE loading dye, and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane, blocked in 5% milk prepared in PBS, and incubated with a mixture of primary

antibodies against TAP (rabbit polyclonal) and yeast Rpl3 (mouse monoclonal), the latter used as an internal control. The molecular weight of TAP-RLuc is ~56 kDa, Rpl3 is ~44 kDa. The blots were washed with 0.1% Tween-20 in PBS and incubated with IRDye-800CW anti-rabbit and IRDye-680RD anti-mouse antibodies. Fluorescent signals were detected using a Typhoon 5 imager at 800 nm and 680 nm.

3. A CFE can also be tested by monitoring the amount of [³⁵S]-Met/Cys-labeled polypeptides synthesized over the course of a translation reaction with endogenous mRNA. Cold methionine and cysteine should be added to a separate translation reaction to serve as a control (Figure 9).

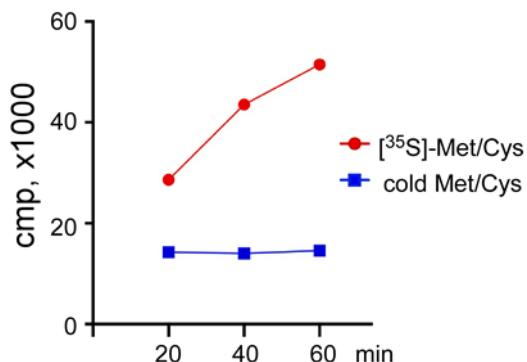


Figure 9. Incorporation of [³⁵S]-Met/Cys into polypeptides synthesized in a CFE.

[³⁵S]-Met/Cys (red) or cold Met/Cys (blue) were added to two CFE-based translation reactions. Three 4 μ L aliquots were collected every 20 min, and proteins were precipitated with TCA. The amount of [³⁵S]-Met/Cys incorporated into polypeptide was estimated by scintillation counting of the counts per minute (CPM) in the TCA precipitates.

4. The integrity of RNAs in CFE can be quickly assessed by SYBR Gold staining and/or Northern blotting (Figure 10). With our CFE preparation protocol, we expect to see only trace amounts of degradation of rRNAs, as indicated by additional faint bands appearing on gels below the main 25S and 18S bands, as compared with RNAs isolated directly from cells using the fast formamide-based extraction method (Shedlovskiy *et al.*, 2017). No degradation of tRNAs or 5.8S rRNA was detected (Figure 10, compare “Cells” lanes and “CFE” lanes).

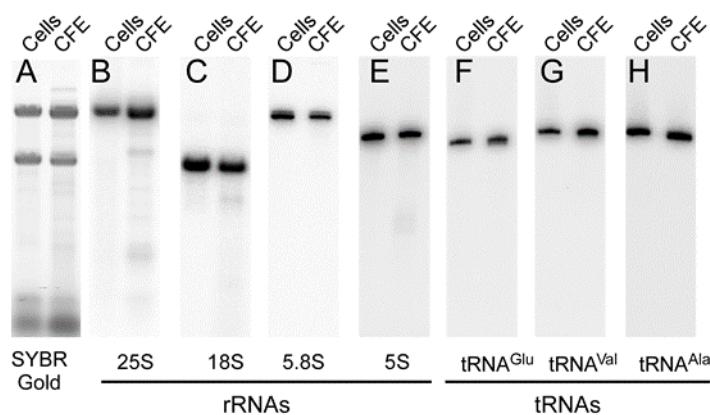


Figure 10. Determination of RNA integrity in a CFE.

RNA derived from a CFE was resolved electrophoretically side-by-side with total RNA extracted from the same yeast strain by a one-step hot formamide method (Shedlovskiy *et al.*, 2017) (lanes labeled “Cells”). Samples were resolved on 1.2% agarose gels containing 1.3% formaldehyde (panels A-C) or on 8M urea-10% polyacrylamide gels (panels D-H). A. RNA resolved on an agarose gel stained with SYBR Gold. B-C. Northern

hybridizations of RNA resolved on agarose gels with [³²P]-labeled probes against 25S and 18S rRNAs. D-H. Northern hybridizations of RNA resolved on polyacrylamide gels with probes against small rRNAs and tRNAs indicated at the bottom.

Notes

1. A critical parameter in the preparation of a yeast CFE is the culture growth conditions. Previously published protocols vary greatly with respect to how yeast culture should be grown. By testing different protocols, we identified culture conditions optimal for the preparation of translation extracts from BY4741 cells. The starting (overnight) yeast culture should reach OD_{λ=600nm} ~10-12; when diluted to OD_{λ=600nm} = 0.8, the culture should be next grown for no longer than 5-6 h at 30°C to reach OD_{λ=600nm} ~2.4-2.5. In our hands, these conditions result in the highest yield and strong translational activity of the yeast lysate.
2. We recommend sterilizing yeast growth media used in this protocol by filtration through a 0.2 µm PES filter instead of autoclaving. Sterilization by filtration better controls the concentration of components in the medium and minimizes variability between CFE preparations.
3. To further improve translational activity, one may wish to modify the genetic background of the yeast strain used to generate the CFE. For example, deletion of the ribosome inactivating factor STM1 was reported to increase the activity of CFEs prepared from *S. cerevisiae* (Brodiazenko *et al.*, 2018). We also found that deletion of the secretory endoribonuclease gene *RNY1* increases the stability of RNAs, such as rRNAs and tRNAs, in yeast lysates (Shcherbik, 2013).
4. The described protocol is optimized for use with translation reporters. To measure absolute amounts of the synthesized protein (in mg/mL), additional steps (such as affinity purification from the CFE translation mixture) are necessary.
5. To design primers for a gene of interest other than Luciferases, we recommend incorporating a T7 promoter sequence (shown in blue, below) in the forward primer sequence upstream of the 5'-end of a coding sequence of a gene. To increase the expression level of a protein in the cell-free translation system described here, the CYC1 5'-UTR enhancer sequence (marked in red below) can be incorporated in the forward primer sequence downstream of the T7 promoter and upstream of the coding region of a gene; however, this step is optional. The Tm for the forward primer should be calculated only for a sequence that anneals to a gene of interest (shown in black in bold lettering). We were successful using the Tm-calculator tool from New England Biolabs: <https://tmcalculator.neb.com/#!/main>. In this tool, a researcher can choose a polymerase that is available in a lab. We were successful using Phusion and Taq polymerases.

5'-ATCCGTAATACGCTCACTATAGGG**AGCTTCACACTAAATTAAATA**ATGXXXXXXXXX-3'

For the reverse primer, a sequence complementary to the 3'-end coding sequence of a gene of interest is sufficient. Tm of the reverse primer should match the Tm of the forward primer. In our experience, Tm of 54-56°C works well for Phusion and Taq polymerases.

Recipes

A. Yeast medium

1. YPD medium
To make 1 L of the liquid YPD medium:
 - a. Mix 20 g of peptone, 10 g of yeast extract, 20 g of dextrose, add deionized H₂O to 1 L.
 - b. Adjust pH to 6.0 with 1 M HCl.
 - c. Sterilize medium by filtration through a 0.2 µm PES filter.
 - d. Filtered YPD can be stored at RT in the dark for up to 2-3 months.
2. YPD-agar plates
Add 20 g of bacto-agar per 1 L of YPD medium; sterilize by autoclaving.

B. Stock solutions

1. 1 M HEPES-KOH, pH 7.6
Take 238.3 g of HEPES acid, add deionized H₂O to 0.9 L, adjust pH to 7.6 with 50% (w/v) KOH, and adjust volume to 1 L; sterilize by autoclaving. Store at room temperature or at 4°C.
2. 2 M KOAc
Take 98.14 g of KOAc, add deionized H₂O to 500 mL, and sterilize by autoclaving. Store at room temperature or at 4°C.
3. 300 mM MgOAc
Take 6.4 g of magnesium acetate tetrahydrate (MgOAc·4H₂O), add deionized H₂O to 100 mL, and sterilize by autoclaving. Store at room temperature or at 4°C.
4. 100 mM MgOAc
Take 2.14 g of magnesium acetate tetrahydrate (MgOAc·4H₂O), add deionized H₂O to 100 mL, and sterilize by autoclaving. Store at room temperature or at 4°C.
5. 1 M DTT
Take 1.5 g of the DTT powder and add deionized H₂O to 10 mL; vortex until you see no crystals. Sterilize the DTT solution by filtration through a 0.2 µm nylon syringe filter. Store at -20°C in 1-ml aliquots.
6. 50% glycerol
Mix 500 mL of glycerol and 500 mL of deionized H₂O, and sterilize the 50% glycerol solution by autoclaving. Store at room temperature or at 4°C.

C. Working solutions

1. Buffer A, 1 L
Mix 30 ml of 1 M HEPES pH 7.6, 50 mL of 2 M KOAc, and 10 mL of 300 mM MgOAc. Use sterile H₂O to adjust the volume to 1 L. Store at 4°C.
2. Buffer A/glycerol, 1 L
Mix 30 mL of 1M HEPES pH 7.6, 50 mL of 2 M KOAc, 10 mL of 300 mM MgOAc, and 400 mL of 50% glycerol solution. Use sterile H₂O to adjust the volume to 1 L. Store at 4°C.
3. Buffer A/mannitol, 1 L
Mix 30 mL of 1M HEPES pH 7.6, 50 mL of 2 M KOAc, 10 mL of 300 mM MgOAc, and 750 mL of deionized H₂O. Weight 85 g of mannitol, add to the solution, and adjust the volume to 1 L. Sterilize by autoclaving. Store at 4°C.
4. Buffer A/mannitol/DTT
Add DTT from the 1 M stock solution to buffer A/mannitol to a final concentration of 2 mM. This solution should be freshly made on the day of the procedure and kept on ice.

Note: For the yeast powder derived from 7-8 g of cell/buffer beads, 20 mL of buffer A/mannitol/DTT is required.

5. Buffer A/glycerol/DTT
Add DTT from the 1M stock solution to buffer A/glycerol to a final concentration of 2 mM. This solution should be freshly made on the day of the procedure and kept on ice.
6. Creatine kinase dilution buffer
Mix 0.1 mL of 1M HEPES pH 7.6 and 0.25 mL of 2 M KOAc. Add 50% sterile glycerol up to 10 ml (9.65 mL). Mix by vortexing and store at -20°C.
7. Creatine kinase solution
Add 0.1 mL of the creatine kinase dilution buffer to 1,000 units of lyophilized creatine kinase to achieve the concentration of 10 units/µL. Store at -20°C with other enzymes.
8. 10× Energy mix
Mix 0.2 mL of 1 M HEPES pH 7.6, 0.1 mL of 100 mM ATP, 0.01 mL of 100 mM GTP, 0.4 mL of 500 mM creatine phosphate, 0.02 mL of 1M DTT, and 0.27 mL of sterile H₂O. The total volume is 1 mL.

- Store at -80°C in 0.03-mL aliquots.
9. FAE solution
Add 0.02 mL of 0.5M EDTA, pH 8.0 to 1 mL of formamide. Store frozen at -20°C.

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Competing interests

The authors declare no competing interests.

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Monitoring Protein Splicing Using In-gel Fluorescence Immediately Following SDS-PAGE

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Abstract

Inteins garner significant interest from both basic and applied researchers due to their unique catalytic abilities. Herein, we describe a protocol for accurately monitoring protein splicing without purification using in-gel fluorescence immediately following Tris-Glycine SDS-PAGE. Following expression in *Escherichia coli*, cells are lysed by sonication, cell supernatants are separated using Tris-Glycine SDS-PAGE, and superfolder GFP (sfGFP) fluorescence is directly visualized within gels. This method is rapid, with sfGFP immediately imaged following SDS-PAGE without staining. Further, sfGFP can be specifically detected in complex samples such as *E. coli* cell supernatants, proteins run at expected masses, and all steps can be performed at ambient temperature. This strategy is broadly applicable beyond the study of protein splicing and can be used for sensitive and specific visualization of superfolder sfGFP-tagged proteins in-gel.

Keywords: In-gel fluorescence, Green fluorescent protein, Intein, Protein splicing

This protocol was validated in: mBio. (2020) DOI: 10.1128/mBio.01403-20.

Background

Phylogenetically widespread in the microbial world, inteins (**intervening proteins**) have generated substantial interest as agents of autocatalysis, drivers of evolution, antibacterial and antifungal targets, and for biotechnological application (Lennon and Belfort, 2017). In the protein splicing reaction, the intein is removed by rearranging two peptide bonds and connecting the surrounding sequences, known as N-and C-exteins, with a peptide bond. While this process can occur in the absence of any external factors, its rate can be highly variable depending on conditions. Recently, compelling evidence has demonstrated that inteins can act as environmental sensors that regulate protein function in response to conditions such as metals, heat, salt, oxidative stress, and DNA damage (Belfort, 2017). Thus, significant interest from both basic and applied communities necessitate the investigation of inteins.

The study of intein catalysis is most often achieved using protein splicing reporters. The only strict requirement of these reporters is the presence of a cysteine, serine, or threonine as the first residue immediately following the intein (Mills *et al.*, 2014). We have utilized a reporter, referred to as MIG (MBP-Intein-GFP), to study the activity of a variety of inteins (Topilina *et al.*, 2015a and 2015b; Kelley *et al.*, 2016 and 2018; Lennon *et al.*, 2018; Green *et al.*, 2019; Woods *et al.*, 2020). In the MIG reporter, the intein (surrounded by 10 native extein residues) is flanked by the Maltose Binding Protein (MBP) as the N-extein and superfolder GFP (sfGFP) as the C-extein (Figure 1). Products with sfGFP attached are detected using in-gel fluorescence immediately following SDS-PAGE. sfGFP-tagged products (those containing a C-extein) allow for the monitoring of all possible intein reactions, including splicing (resulting in fluorescent ligated exteins), N-terminal cleavage (resulting in fluorescent intein-C-extein), and C-terminal cleavage (resulting in fluorescent C-extein) (Figure 1). Depending on the intein and reaction conditions, not all products may be detected. The major advantages of our sfGFP-based reporter, compared to other protein splicing reporters, is that there is no need for purification following expression and products can be visualized without staining.

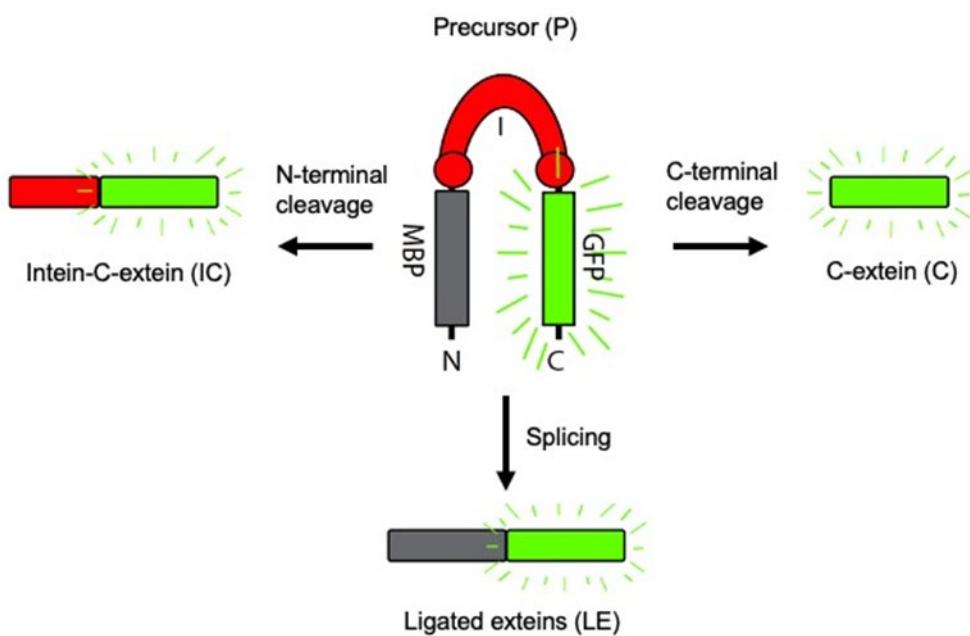


Figure 1. Maltose binding protein-Intein-sfGFP (MIG) protein splicing reporter.

Products of splicing, N-terminal cleavage, and C-terminal cleavage that are visible (*i.e.*, sfGFP-containing) using in-gel fluorescence are shown for each reaction.

Moreover, the use of in-gel fluorescence is broadly applicable beyond the study of protein splicing. This in-gel fluorescence protocol can be used to accurately examine the size of any sfGFP-tagged protein expressed in *E. coli* without purification. For example, this strategy could be used to study the localization of proteins in different cellular fractions, such as the cytoplasm, periplasm, and membrane of *E. coli*. Similar procedures have been described for visualization of GFP-tagged proteins. The protocol described herein differs as it allows visualization without staining (Pristov *et al.*, 2015) and does not require running gels at 4°C (Bird *et al.*, 2015). Several critical parameters relating to sfGFP-detection are also addressed. As GFP structure is required to maintain its fluorescent signal, it is critical that samples are not heated prior to SDS-PAGE (Figure 2). Further, we find that the use of Tris-Glycine rather than Bis-Tris gels improves sensitivity (Figure 2).

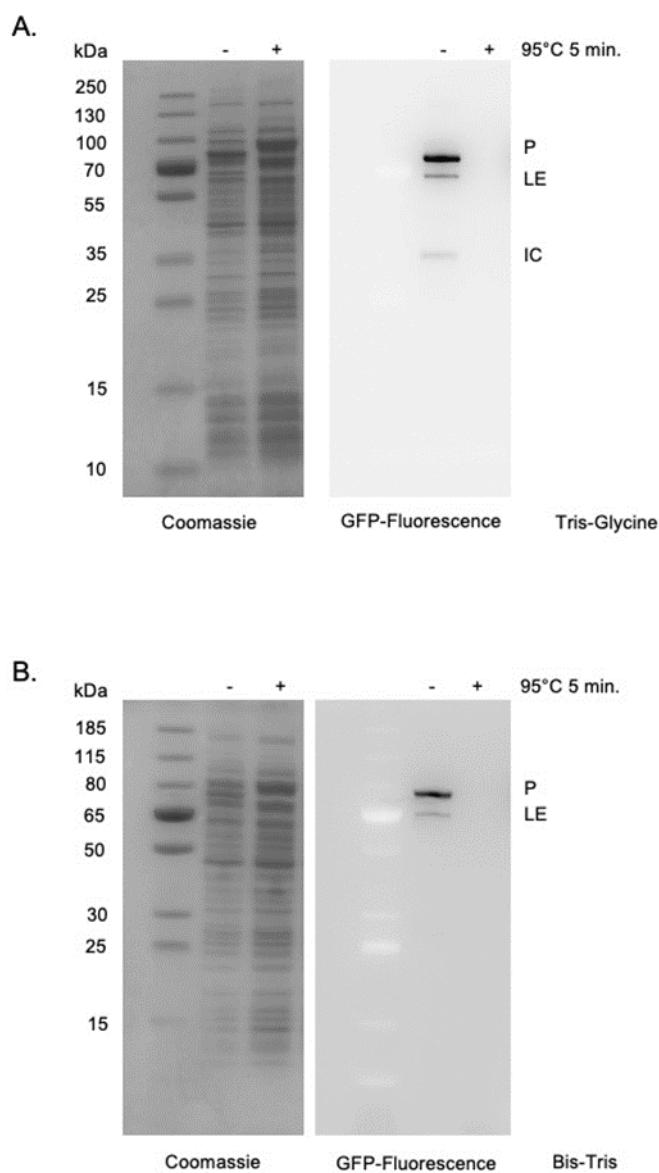


Figure 2. sfGFP detection within SDS-PAGE.

Tris-Glycine gels (Panel A) are more sensitive than Bis-Tris gels (Panel B) for detecting in-gel sfGFP signals as indicated by stronger signal, lower background, as well as detection of the minor product (IC) only in Tris-Glycine gels. sfGFP fluorescence is specifically detected in cell supernatants without purification. Heating samples at 95°C for 5 min results in a loss of fluorescent signal. Left, Coomassie stained. Right, sfGFP signal.

Expected molecular weights of products are as follows: Precursor (P) 88.5 kDa; Ligated exteins (LE) 74.0 kDa; Intein-C-extein (IC) 42.6 kDa.

Materials and Reagents

1. 10, 20, and 1,000 μ L Pipette tips (USA Scientific, Inc. TipOne, catalog numbers: 1111-3800, 1120-1810, and 1111-2820)
2. 1.5 mL tubes (Eppendorf, catalog number: 022363204)
3. 50 mL conical tubes (Corning, catalog number: 352070)
4. Gel loading tips (USA Scientific, catalog number: 1022-0000)
5. Culture tube (Fisher Scientific, catalog number: 14-961-31)
6. 10 mL syringe (BD, catalog number: 309604)
7. 0.22 μ m syringe filter (Fisher Scientific, catalog number: 09-720-3)
8. Mini-PROTEAN TGX Precast Protein Gels, Tris-Glycine, 8-16% (Bio-Rad Laboratories, catalog number: 4561106)
9. 4 \times Laemmli Sample Buffer (Bio-Rad Laboratories, catalog number: 1610747)
10. 10 \times Tris/Glycine/SDS electrophoresis buffer (Bio-Rad Laboratories, catalog number: 1610732)
11. MIG (or sfGFP) expression strain (available upon request)
12. Tris, 1.0 M buffer solution, pH 7.5, 0.2- μ m filtered (Alfa Aesar, catalog number: J62993)
13. Tris, 1.0 M buffer solution, pH 8.5, 0.2- μ m filtered (Alfa Aesar, catalog number: J61038)
14. Glycerol (Fisher Scientific, catalog number: BP229)
15. Zinc acetate dihydrate (Fisher Scientific, catalog number: S25634)
16. LB broth base (Difco, catalog number: 244610)
17. IPTG (Goldbio, catalog number: 12481C25)
18. Chloramphenicol (Sigma, catalog number: C0378)
19. MIG Buffer (see Recipes)
20. 1 \times Tris/Glycine/SDS Running Buffer (see Recipes)
21. 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG) (see Recipes)
22. 25 mg/mL Chloramphenicol stock solution (*i.e.*, 1,000 \times , see Recipes)
23. LB medium (see Recipes)
24. 10 mM Zinc acetate solution (see Recipes)

Equipment

1. Cell Sonicator (Heat Systems, model: XL-2020)
2. FB300 Power Supply (Fisher Scientific, catalog number: S65533Q)
3. Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels, 4-gel (Bio-Rad Laboratories, catalog number: 1658004)
4. Amersham Imager 600 (GE, catalog number: 29-0834-61)
5. P20 Pipetman (Gilson, catalog number: FA10003M)
6. P1000 Pipetman (Gilson, catalog number: FA10006M)
7. Microcentrifuge (Eppendorf, catalog number: 5420000113)
8. Centrifuge (Fisher Scientific, catalog number: 75005194)
9. Benchtop Refrigerated Shaking Incubator (Thermo Scientific, catalog number: SHKE4000)

Software

1. ImageJ (software free to download at <https://imagej.nih.gov>)

Procedure

A. Protein expression (Figure 3)

1. Prepare, under sterile conditions, a saturated overnight culture of the MIG (or sfGFP) expression strain by inoculating a culture tube containing 5 mL LB media and 5 μ L of 25 mg/mL (1,000 \times) chloramphenicol stock solution.
2. Incubate at 37°C and 250 RPM for approximately 16 h.
3. Under sterile conditions, dilute 1 ml of the overnight culture into 50 mL LB media and 50 μ L 25 mg/mL (1,000 \times) chloramphenicol stock solution.
4. Incubate at 37°C and 250 RPM for 90 min.
5. Under sterile conditions, add 50 μ L 1 M IPTG (final concentration of 1 mM IPTG).
6. Incubate at 37°C and 250 RPM for 60 min.

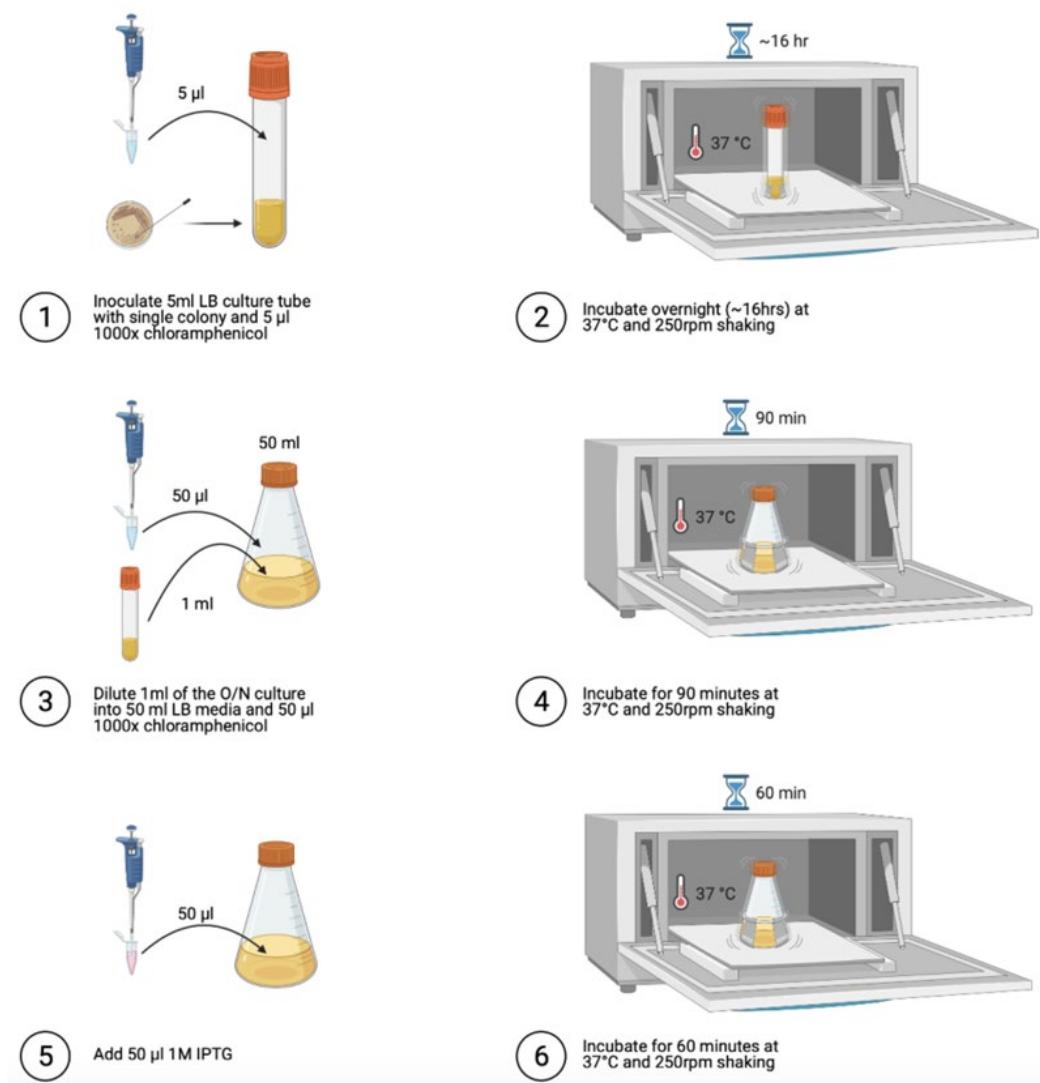


Figure 3. Steps for protein expression. Image generated using Biorender.

B. Cell lysis (Figure 4)

1. Transfer to 50 mL conical tube.
2. Spin to pellet at $4,000 \times g$ for 10 min at room temperature ($\sim 22^\circ\text{C}$).
3. Discard supernatant without disturbing pellet.
4. Resuspend pellet in 1 mL MIG buffer.
5. Sonicate on ice (35% intensity) for 10 s on, then 10 s off for 2 min.
6. Transfer lysed cells into 1.5 mL tube.
7. Spin at 4°C and $16,000 \times g$ for 10 min.
8. Without disturbing pellet, transfer supernatant into 1.5 mL tube and place on ice.



Figure 4. Steps for cell lysis.

Image generated using Biorender.

C. Protein splicing

1. Pipette 45 μL of supernatant into a 1.5 mL tube, add 15 μL 4 \times Laemmli dye and mix, and place at -20°C .

Note: This is time point 0.

2. Pipette 495 μL of supernatant and 5 μL ddH₂O into a 1.5 mL tube and incubate under foil at room temperature ($\sim 21^\circ\text{C}$).
3. In another 1.5 mL tube, pipette 99 μl supernatant and 1 μl 10 mM zinc acetate dihydrate and incubate under foil at room temperature ($\sim 21^\circ\text{C}$).
4. At 1, 2, 4, 8, and 24 h time points, take 45 μL from the tube without zinc acetate dihydrate and pipette into a new 1.5 mL tube, add 15 μL 4 \times Laemmli dye and mix, and place at -20°C .

*Note: The rate of protein splicing is highly variable depending on the intein and incubation conditions. Adjust time points accordingly. These conditions are for the *Mycobacterium smegmatis DnaB1* intein.*

5. At the 24-hour time point, take 45 μ L from the tube with supernatant and zinc acetate dihydrate and pipette into new 1.5 mL tubes, add 15 μ L 4 \times Laemmli dye and mix, and place at -20°C.

D. Tris-Glycine SDS-PAGE

1. Thaw samples at room temperature.
2. Do not heat samples prior to electrophoresis.

Note: Heating will denature sfGFP and result in loss of fluorescent signal (Figure 2).

3. Add 1 \times running buffer to electrophoresis tank.
4. Insert Tris-Glycine gel into the electrophoresis gel holder and add gel holder to tank.
5. Add 1 \times running buffer inside of electrophoresis gel holder to cover gel wells.
6. Load 4 μ L ladder and 6 μ L of each sample.
7. Connect tank to power supply and run gel at 200 V for 40 min.
8. Remove gel from plastic shell and place in ddH₂O.

E. Image Gel

Measure in-gel fluorescence using a Amersham 600 Imager or an equivalent imager capable of detecting sfGFP fluorescence (Figure 5).

1. Lay gel flat on imaging tray.
2. Place tray in imager.
3. Set imager to Epi-RGB: Blue light (460 nm, Filter Cy2).
4. Set exposure to auto with high dynamic range.
5. Save picture of gel as tiff or jpeg. Relative fluorescence can be calculated by densitometry using ImageJ.

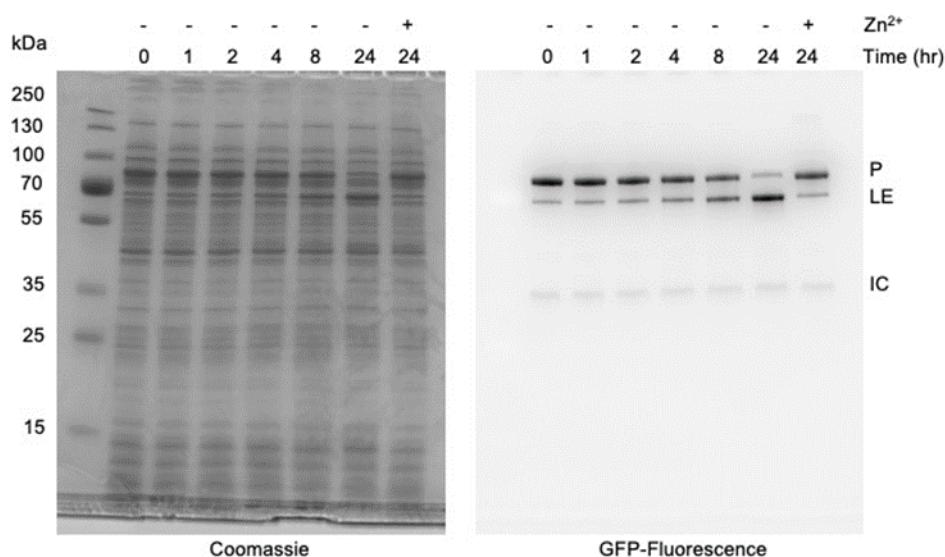


Figure 5. Monitoring protein splicing of *Mycobacterium smegmatis* DnaBi1 intein using in-gel fluorescence with Tris-Glycine SDS-PAGE.

Presence of 100 μ M zinc acetate inhibits splicing. Left, Coomassie stained. Right, sfGFP signal. sfGFP-tagged products are indicated as Precursor (P), Ligated exteins (LE), and Intein-C-extein (IC).

Recipes

1. MIG Buffer

2.5 mL Tris, 1.0 M buffer solution, pH 7.5, 0.2 µm filtered
2.5 mL Tris, 1.0 M buffer solution, pH 8.5, 0.2 µm filtered
10 mL Glycerol
85 mL ultrapurified H₂O

2. 1× Tris/Glycine/SDS running buffer (25 mM Tris, pH 8.3, 0.1% SDS, 192 mM Glycine)

100 mL 10× Tris/Glycine/SDS electrophoresis buffer
900 mL ultrapurified H₂O

3. DNA precipitation solution 10 mM zinc acetate

109 mg zinc acetate dihydrate
50 mL ultrapurified H₂O

4. LB Medium

25 g LB broth base
1,000 mL ddH₂O
Autoclave to sterilize

5. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG)

2.38 g IPTG
10 mL ultrapurified H₂O
Place in 10-mL syringe and pass through 0.22-µm filter to sterilize
Aliquot in 1.5-mL tubes and store at -20°C

6. 25 mg/mL chloramphenicol stock solution

250 mg chloramphenicol
10 mL 100% ethanol
Place in 10-mL syringe and pass through 0.22-µm filter to sterilize
Aliquot in 1.5-mL tubes and store at -20°C

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Tomato Stem Injection for the Precise Assessment of *Ralstonia solanacearum* Fitness in *Planta*

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#Contributed equally to this work

Abstract

Ralstonia solanacearum is a soil-borne pathogen with worldwide distribution that causes bacterial wilt disease in more than 250 plant species. *R. solanacearum* invades plants through the roots, reaches the vascular system, and colonizes the whole plant by moving through the xylem, where it eventually replicates rapidly, causing plant death. Usual assays to measure the virulence of *R. solanacearum* under laboratory conditions rely on soil-drenching inoculation followed by observation and scoring of disease symptoms. Here, we describe a protocol to assess the replication of *R. solanacearum* following injection into tomato stems. This protocol includes four major steps: 1) growth of tomato plants; 2) *R. solanacearum* injection into tomato stems; 3) collection of tomato xylem samples and bacterial quantitation; and 4) data analysis and representation. This method bypasses the natural penetration process of the pathogen, thus minimizing variation associated with stochastic events during bacterial invasion, and provides a sensitive and accurate measurement of bacterial fitness inside xylem vessels.

Keywords: *Ralstonia solanacearum*, Stem injection, Tomato, Virulence, Fitness, Bacterial wilt

This protocol was validated in: Cell Host Microbe. (2020) DOI: 10.1016/j.chom.2020.07.003.

Background

Ralstonia solanacearum is the causal agent of bacterial wilt disease in more than 250 plant species including important crops, such as tomato, potato, pepper, and eggplant, among others, and is considered one of the most dangerous plant pathogens in the world (Mansfield *et al.*, 2012). *R. solanacearum* is a soil-borne pathogen that enters plants through the roots, using wounds, root tips, and secondary root emergence points as penetration sites; it then progresses via the root cortex, finally reaching the vascular system (Xue *et al.*, 2020). In susceptible plants, *R. solanacearum* moves through the xylem vessels to colonize the whole plant; eventual replication may lead to populations up to 10^{10} bacteria per gram of plant tissue in diseased plants, which will block water and nutrient flow in the vascular system and eventually cause plant wilting and death (Peeters *et al.*, 2013). Multiple experimental methods to assess *R. solanacearum* virulence in plants under laboratory conditions have been described previously (Morel *et al.*, 2018). The most widely used assay relies on soil-drenching of plant roots with *R. solanacearum* suspensions, followed by observation of the resulting wilting symptoms over time (Morel *et al.*, 2018). Other assays involve bypassing the root penetration process by introducing the bacterial suspensions directly into plant tissues (either leaves or stems) and provide more accurate assessments of bacterial replication (Macho *et al.*, 2010; Morel *et al.*, 2018; Yu and Macho, 2021).

In this protocol, we provide a detailed description of a method aimed at quantitating bacterial fitness in tomato xylem vessels by direct injection into the stem using a microsyringe; given the subsequent fast bacterial replication and plant colonization, we assume that a substantial proportion of this inoculum reaches the xylem vessels. At the desired time after inoculation, samples are taken from 2 cm above the inoculation site by collecting xylem sap from an excised stem section. Bacterial quantitation in this xylem sap provides a clean measurement of bacterial fitness in the plant vascular system. Like other Gram-negative bacterial pathogens, *R. solanacearum* requires a type III secretion system to inject type III effector (T3E) proteins into host cells and cause disease. Since T3E activities often contribute redundantly to the development of disease, it is generally difficult to determine their involvement in virulence; however, we have recently used this method to detect the virulence attenuation of different *R. solanacearum* knockout mutants lacking individual effectors (Xian *et al.*, 2020; Yu *et al.*, 2020) and to analyze bacterial pathogenicity following chemical treatment of plant tissues (Wang *et al.*, 2021), revealing a simple, versatile, and powerful assay for the study of *R. solanacearum*-plant interactions.

Materials and Reagents

1. Microcentrifuge tubes (1.5 mL and 2 mL) (BBI, catalog number: F600620-0001)
2. Pipette tips
3. Plastic Petri dishes (90 mm diameter)
4. Jiffy pots (41 mm diameter) (Jiffy International, Kristiansand, Norway)
5. Spectrophotometer plastic cuvettes (BRAND, catalog number: 759015)
6. Syringe filter (0.45 µm) (Millex, Millex®-HV, catalog number: SLHV033RB)
7. Tomato seeds (*Solanum lycopersicum* cv. Moneymaker)
8. Standard potting soil (Pindstrup, catalog number: 1034593214)
9. Vermiculite (generic)
10. Distilled sterile water
11. 75% ethanol (Sinopharm Chemical Reagent Co, SCR®, catalog number: 801769610)
12. Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, catalog number: T8877-5G)
13. Glucose (Sinopharm Chemical Reagent Co, SCR®, catalog number: 63005518)
14. Bacto peptone (BD, catalog number: 211677)
15. Yeast extract (OXOID, catalog number: LP0021)
16. Casein hydrolysate (Casamino acids) (Sigma-Aldrich, catalog number: 22090-500G)
17. Agar (Sinopharm Chemical Reagent Co, SCR®, catalog number: 10000561)
18. Phi medium (see Recipes)
19. 1% (W/V) TTC solution (see Recipes)

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20. 20% (W/V) glucose solution (see Recipes)

Equipment

1. Micro sample syringe 10 μL (Sangon Biotech, catalog number: E718LD0023)
2. Sterile scalpel
3. EasySpiral® Automatic Serial Diluter and Plater (optional) (Interscience, Easyspiral®, catalog number: 412000)
4. Plant growth chamber (Percival, model: I-36VL)
5. Autoclave (SANYO, model: MLS-3780)
6. Centrifuge (Eppendorf, model: Centrifuge 5424)
7. pH meter (Sartorius, model: PB-10)
8. Petri dish incubator at 28°C (Panasonic, model: MIR-262-PC)
9. Tube incubator (shaker) at 28°C (Eppendorf, New BrunswickTM, catalog number: m1324-0006)
10. NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000c)
11. Water distiller/sterilizer (Millipore, model: Milli-Q integral 10L)
12. Vortex (Scientific Industries, model: Vortex-Genie 2, catalog number: S1-0246)

Software

1. GraphPad Prism 7 (<https://www.graphpad.com/scientific-software/prism/>)Excel 2016 (Microsoft Corporation)

Procedure

A. Growing tomato plants

1. Germinate tomato seeds (*Solanum lycopersicum* cv. Moneymaker) in a 1:2 mixture of standard potting soil and vermiculite. Grow seedlings for one week in a growth chamber (25°C, 16 h light/8 h dark photoperiod, 130 mE $\text{m}^{-2} \text{s}^{-1}$, 65% humidity) (Figure 1A).
2. Soak Jiffy pots thoroughly with tap water without fertilizer (an excess of fertilizer may cause stress to the tomato seedlings) (see Note 1). Transfer tomato seedlings to individual water-soaked jiffy pots containing a pre-packed soil mixture (Figure 1B) (see Note 1). Grow tomato plants in the same growth chamber for another three weeks (Figure 1C) (see Note 2). Approximately 6-8 plants per treatment are recommended to obtain robust results.



Figure 1. Growth of tomato seedlings in Jiffy pots.

A. Germination of tomato seeds (*Solanum lycopersicum* cv. Moneymaker) in a 1:2 mixture of standard potting soil and vermiculite. B. Transferring tomato seedlings to individual water-soaked jiffy pots containing a pre-packed soil mixture. C. Four-week-old tomato plants ready for *Ralstonia solanacearum* inoculation.

B. Preparation of *R. solanacearum* inoculum

1. Prepare solid and liquid phi medium (see Recipes) following standard procedures (Yu and Macho, 2021).
2. Streak out *R. solanacearum* strains on solid phi medium plates containing TTC and glucose (see Recipes; Yu and Macho, 2021) and prepare a bacterial suspension as previously described (Yu and Macho, 2021).
3. Dilute the final bacterial suspension in distilled water to an $\text{OD}_{600} = 0.1$, which corresponds to approximately 10^8 CFU/ml (Morel *et al.*, 2018). Perform serial dilutions of the bacterial suspension using distilled water to reach 10^6 CFU/ml in a volume of 1 mL in a sterile 2-ml microcentrifuge tube (see Note 3) (Figure 2). Consider the possibility of testing the virulence of different doses of your specific strains under your specific experimental conditions. Under our conditions, we selected 10^6 CFU/mL as a suitable concentration to allow gradual but reproducible bacterial replication inside tomato stems during the first 3 days post-inoculation (dpi) without causing the development of necrotic symptoms during the experiment (Figure 3).

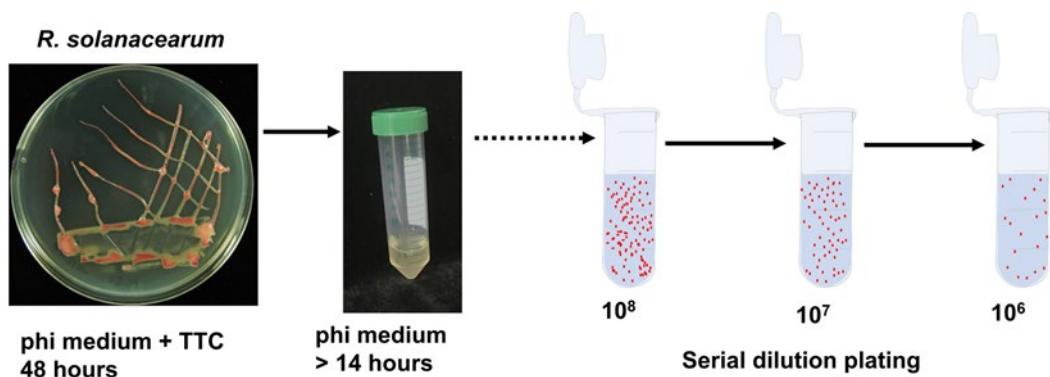


Figure 2. Preparation of *R. solanacearum* inoculum

C. Inoculation (stem injection) and sampling of xylem sap

1. Using a 10- μL microsyringe, inject 5 μL bacterial suspension into the stems of 4-week-old tomato plants (see Note 4). The inoculation site should be approximately 2 cm below the cotyledon emergence site (Figure 3A and 3B). When performing stem injection, use an angle of approximately 30° between the syringe and the stem to facilitate injection of the bacterial suspension.
2. Place the inoculated tomato plants in a growth chamber (28°C , 12 h light/12 h dark, $130 \text{ mE m}^{-2} \text{ s}^{-1}$, 75% humidity) for 36-72 h until subsequent sampling at the appropriate time points.
3. Transect the stem with a sterile scalpel just below the cotyledon emergence site (Figure 3C).
4. Using two fingers, gently squeeze the lower part of the tomato stem to allow the xylem sap to emerge and then collect 2.5 μl xylem sap using a 2.5- μL pipette (see Note 5) (Figure 3D).

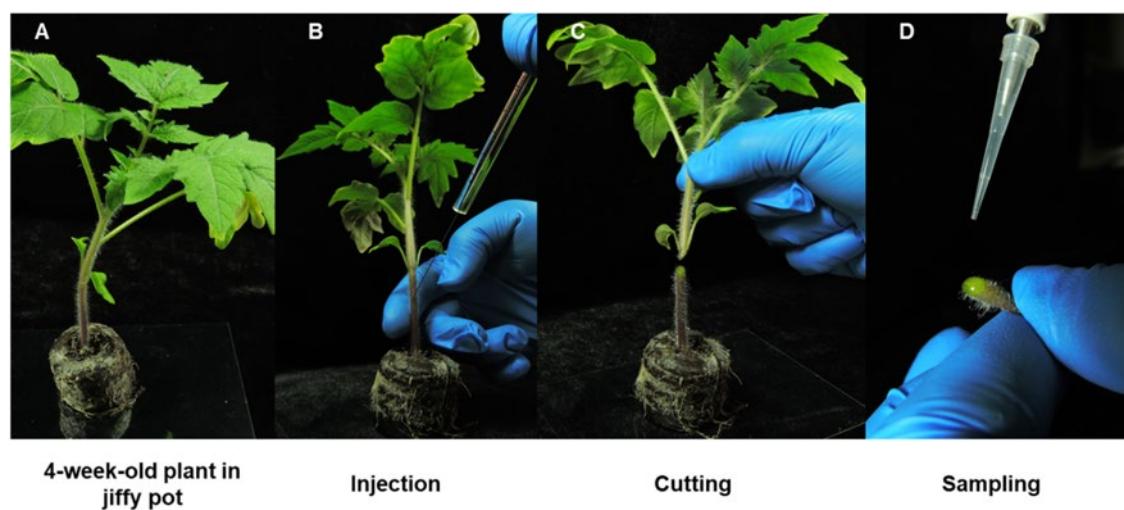


Figure 3. Stem injection and sampling of xylem sap.

A. Four-week-old tomato plants in Jiffy pots. B. Using a 10- μ L microsyringe, inject 5 μ L bacterial suspension into the stems of 4-week-old tomato plants. C. Transect the tomato stems with a sterile scalpel just below the cotyledon emergence site. D. Collect 2.5 μ L xylem sap at the cutting site using a micropipette.

D. Bacterial quantitation

1. Dissolve the xylem sap in 1 mL sterile water in a sterile 1.5-mL microcentrifuge tube (Figure 4A).
2. Dilute and spread bacteria (50 μ L) on phi medium plates using standard procedures, as previously described (Yu and Macho, 2021). Spreading can be performed manually using a sterile spreader, plastic beads, or the bottom of a 1.5-mL microcentrifuge tube, or using a spiral plater (Yu and Macho, 2021).
3. Incubate the plates upright at 28°C for 2 days. Count the colonies and process the data (Figure 4B) (see Note 6).

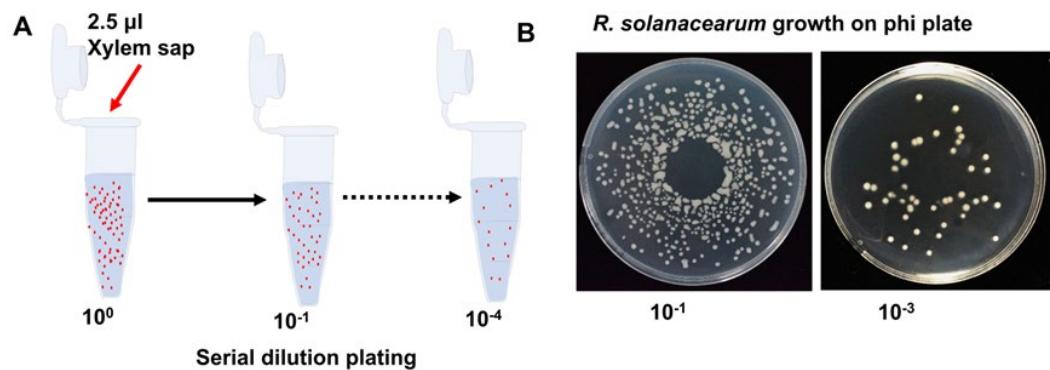


Figure 4. Preparation of serial dilutions and bacterial spreading.

A. Dissolve the xylem sap containing *Ralstonia* in 1 mL sterile water in a sterile 1.5-mL microcentrifuge tube; dilute the suspensions by 10 to 10⁴ times. B. Spread the diluted bacterial suspensions (we tried to spread 10⁻¹ or 10⁻³) on phi medium plates using standard procedures, as previously described (Yu and Macho, 2021). Incubate the plates upright at 28°C for 2 days and count the colonies on solid phi medium plates. Photos in B show the usual colony pattern after plating using a spiral plater (Yu and Macho, 2021).

Data analysis

1. Using the number of colonies on each readable plate, calculate bacterial numbers in the sampled xylem sap. If plating was performed manually, apply the following formula:
 $R. solanacearum$ numbers in xylem sap (CFU/ml) = colony number $\times 20 \times 10^3 \times 10^{(\text{dilution factor})}/2.5$

Note: "Colony number": number of colonies on the readable plate. "20" corresponds to the result of dividing 1,000 μl final diluted bacteria between 50 μl bacterial solution spread on the plate. "10³" corresponds to the conversion of CFU/ μl to CFU/ml. "2.5" corresponds to the 2.5 μl xylem sap taken as a sample and dissolved in 1 ml water. "10^(dilution factor)" corresponds to the additional serial dilution of the xylem sap.

2. If plating was performed using a spiral plater, follow additional calculations as previously described (Yu and Macho, 2021).
3. Repeat the experiment at least 3 times. Input the final data to GraphPad Prism 7 or a similar program for statistical analysis. To compare results individually, perform a Student's t-test and report the mean value, SEM, and P value to indicate the statistical significance of the differences. Results are better displayed using a logarithmic scale and the individual data points together with the mean \pm SEM (Figure 5).

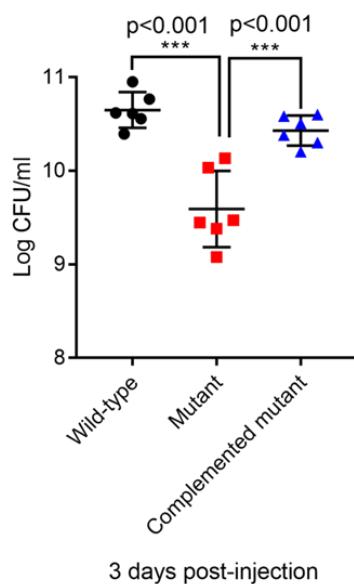


Figure 5. Representation of the growth of *R. solanacearum* GMI1000 wild-type, mutant, and complemented strain in tomato stems.

Different colors represent different strains, and horizontal bars represent mean values ($n = 6$ plants per strain). Data are represented as the mean \pm SEM. Asterisks indicate significant differences (** $P < 0.0001$, t-test). The graph was plotted using GraphPad Prism 7.

Notes

1. If the tap water has a high mineral content or contains excessive impurities, purified water can also be used.
2. Grow no more than 24 tomato plants in each tray (29 \times 20 cm), and separate the plants to avoid intertwined roots among individual plants. Water the plants normally once every two days, and keep the jiffy pots wet.
3. For serial dilution, use a 1:10 dilution for each step.

4. Optionally, move the tomato plants to a growth chamber (28°C, 12 h light/12 h dark, 130 mE m⁻² s⁻¹, 75% humidity) 12 h before *R. solanacearum* injection to minimize interference caused by changing the growth chamber after inoculation. This does not make a difference under our conditions.
5. For tomato sampling, remove the oozing sap gently with sterile paper, and clamp the stem top with a finger to push the xylem sap out for sampling.
6. Waste disposal: Autoclave all the material used in this experiment before disposal to avoid releasing *R. solanacearum* into the environment. Clean the work bench and flow hood with 75% alcohol after the experiment.

Recipes

1. Phi medium

10 g Bacto peptone
1 g Yeast extract
1 g Casamino acids

Add water to reach 1 L. To prepare solid medium, add 15 g agar to 1 L medium and autoclave.

2. 1% (W/V) TTC solution

Dissolve TTC in distilled water

Note: Store 1% TTC solution at room temperature or 4°C in a dark environment.

3. 20% (W/V) glucose solution

Glucose should be dissolved in distilled water and autoclaved. Store at 4°C.

*Note: Add 1% TTC and 20% glucose when preparing solid Phi medium plates to induce pink *R. solanacearum* colonies.*

Acknowledgments

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Competing interests

The authors have no competing interests to declare.

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Isolation and Characterization of Membrane Vesicles from *Lactobacillus* Species

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Abstract

Throughout their life cycle, bacteria shed portions of their outermost membrane comprised of proteins, lipids, and a diversity of other biomolecules. These biological nanoparticles have been shown to have a range of highly diverse biological activities, including pathogenesis, community regulation, and cellular defense (among others). In recent publications, we have isolated and characterized membrane vesicles (MVs) from several species of *Lactobacilli*, microbes classified as commensals within the human gut microbiome (Dean *et al.*, 2019 and 2020). With increasing scientific understanding of host-microbe interactions, the gut-brain axis, and tailored probiotics for therapeutic or performance increasing applications, the protocols described herein will be useful to researchers developing new strategies for gut community engineering or the targeted delivery of bio-active molecules.

Keywords: Membrane vesicles, Lactic acid bacteria, *Lactobacillus acidophilus*, *Lactobacillus plantarum*

This protocol was validated in: Sci Rep. (2019) DOI: 10.1038/s41598-018-37120-6.

Graphical Abstract:

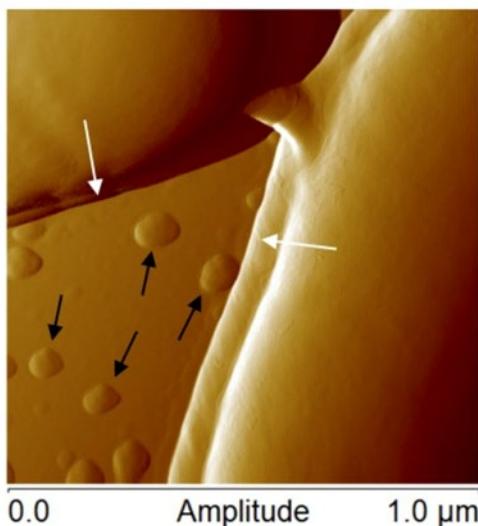


Figure 1. Atomic force microscopic image of *Lactobacillus casei* ATCC 393 bacteria margins (white arrows) and membrane vesicles (black arrows)

Background

The membranes of all cells are dynamic structures, the biomolecule composition of which is constantly changing as cells respond to environmental stimuli, alter protein and lipid composition, release waste products, take in nutrients, and perform many other cellular processes (Vererb *et al.*, 2003; Watson, 2015). Throughout cellular life cycles, fragments of the outermost membrane are often shed as nanosized particles. In bacteria, these structures are often referred to as membrane vesicles (MVs) or outer membrane vesicles (OMVs), which will be referred to as MVs throughout for simplicity (a representative example of MVs from *Lactobacillus casei* is shown in Figure 1). As interest in these biological nanoparticles has grown in recent years, researchers have shown that MVs have broad biological activities, including host-microbe interactions, gene transfer, and community regulation (Kulp and Kuehn, 2010; Caruana and Walper, 2020).

Bacterial MVs have shown significant promise for applications such as vaccine development and potential therapeutic applications. Naturally occurring OMVs from Gram-negative bacteria have shown significant promise in the development of vaccines for bacteria such as *Neisseria meningitidis* and *Burkholderia pseudomallei*, pathogens that have proven challenging to vaccine and therapeutic development alike (Holst *et al.*, 2009; Nieves *et al.*, 2014). Recently, research groups have also shown that the MVs from some commensal bacteria can also modulate responses from host immune systems and stimulate host neurological systems (Mata Forsberg *et al.*, 2019; Molina-Tijeras *et al.*, 2019; Rodovalho *et al.*, 2020). With growing capabilities in synthetic biology, the potential uses of MVs are steadily increasing as researchers have developed engineering strategies that allow for modification of genetic and cellular pathways to control the composition of nascent MVs. These efforts have led to new biomaterials for therapeutic applications, environmental decontamination, and other purposes (Alves *et al.*, 2018; Qing *et al.*, 2019).

The classification of lactic acid bacteria (LAB) encompasses several genera of bacteria with similar characteristics of acid-tolerance and fermentation capabilities. Many LAB are classified as generally regarded as safe (GRAS) and have been used in the production of food products for centuries. Additionally, several LAB have been recognized as beneficial to their host and are studied for their health benefits as probiotics leading to a large commercial market for probiotic supplements and foods. While live-bacterial cultures are the most commonly used form of probiotics,

the potential for engineering or enriching for specific cellular components has led researchers to explore the use of purified MVs for controlled therapeutic applications (Seo *et al.*, 2018; Molina-Tijeras *et al.*, 2019; Dean *et al.*, 2020). There are numerous protocols for the purification of MVs from eukaryotic cells and Gram-negative bacteria, which have been the primary focus of MV research (Klimentova and Stulik 2015; Alves *et al.*, 2017; Dauros Singorenko *et al.*, 2017). Recently, there has been growing interest in the MVs of gut bacteria and the roles they may play in host and community interactions. LAB are Gram-positive bacteria and therefore have a significantly different membrane and peptidoglycan structure as compared to Gram-negative bacteria. While this may not specifically contribute to biophysical differences between Gram-negative and Gram-positive MVs, it has been shown that the MVs of some LAB species have a bimodal size distribution with an abundant population of smaller MVs in the 10–50 nm size range (Dean *et al.*, 2019). Here, we describe protocols for the isolation and characterization of MVs that have proven successful for numerous LAB species. MVs are isolated from overnight LAB cultures via ultracentrifugation and then analyzed for concentration and relative size distribution using a NanoSight nanoparticle tracking instrument. Dynamic light scattering (DLS) is used as another way to measure size distribution and also to measure zeta potential (surface charge). Then, the protein content of MVs can be examined both qualitatively using SDS-PAGE and specifically by using mass spectrometry for proteomic analysis (schematic shown in Figure 2). While this manuscript describes work we have employed for LAB, these protocols could also be used for the isolation of MVs from a variety of microbial species.

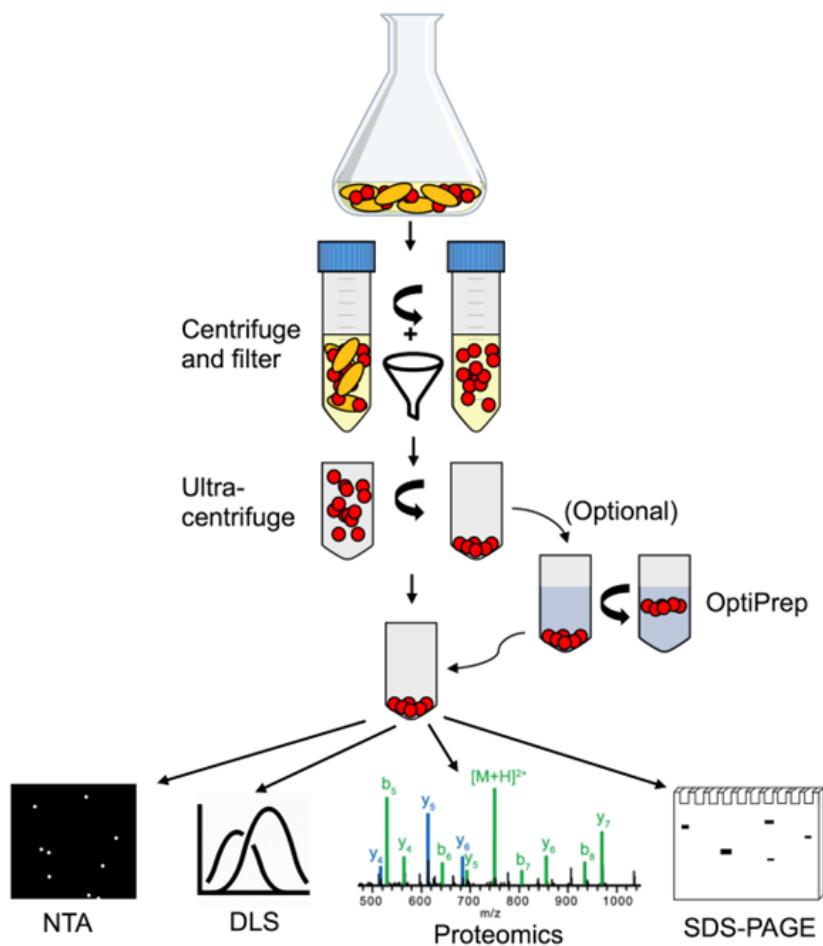


Figure 2. Schematic overview of the membrane vesicle isolation and characterization process.

MVs are purified from *Lactobacillus* cultures initially via centrifugation and filtration, removing cells and large cellular debris. From the filtered supernatant, MVs are isolated by ultracentrifugation. Gradient centrifugation using OptiPrep medium can be applied to isolated MVs as an additional purification step. Purified MVs can then be characterized by nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), SDS-PAGE

protein gels, shotgun proteomics, or other methods.

Materials and Reagents

1. AnaeroJar™ 2.5 L jars (Oxoid®, ThermoFisher Scientific, catalog number: AG0025A)
2. AnaeroGen™ anaerobic gas generation sachets (Oxoid®, Thermo Fisher Scientific, catalog number: AN0025A)
3. Resazurin anaerobic indicator strips (Oxoid®, Thermo Fisher Scientific, catalog number: BR0055B)
4. 100 mm sterile Petri dishes (Fisherbrand, Thermo Fisher Scientific, catalog number FB0875712)
5. Pyrex® Erlenmyer flasks 250 mL (Corning™, Thermo Fisher Scientific, catalog number: CLS4980250)
6. 50 mL conical centrifuge tubes (Corning™ Falcon, Thermo Fisher Scientific, catalog number: 14-432-22)
7. 0.45 µm syringe filters (J. G. Finneran, catalog number: FEC0425PC)
8. 30 mL sterile syringes (BD Slip tip sterile syringe; Thermo Fisher Scientific, catalog number: BD 302833)
9. 38.5 mL ultracentrifuge tubes (Thinwall Ultra-Clear tubes; Beckman Coulter, catalog number: 344058)
10. 5 mL ultracentrifuge tubes (Thinwall Ultra-Clear tubes; Beckman Coulter, catalog number: 344057)
11. 1 mL needle-less syringes (Henke Sass Wolf, catalog number: 4010.200V0)
12. 1.5 ml centrifuge tubes (Thermo Fisher Scientific, catalog number: P190411)
13. Disposable cells compatible with ZetaSizer Nanoseries (Malvern Panalytical, catalog number: DTS 1070)
14. *Lactobacillus* species, *i.e.*,
Lactobacillus acidophilus (ATCC 53544)
Lactobacillus casei (ATCC 393)
Lactobacillus reuteri (ATCC 23272)
Lactobacillus plantarum (ATCC BAA-793)
15. de Man, Rogosa, and Sharpe (MRS) media (Sigma-Aldrich, catalog number: 69966-500G, prepare according to the manufacturer's instructions)
16. Tween® 80 (Sigma Aldrich, catalog number: P8074)
17. 4-15% Mini-PROTEAN® TGX Pre-cast protein gels (Bio-Rad, catalog number: 4561085)
18. GelCode™ Blue Stain Reagent (Thermo Fisher Scientific, catalog number: PI24590)
19. 10× phosphate-buffered saline (Thermo Fisher Scientific, catalog number: AM9625)
20. 4× Laemmli sample buffer (Bio-Rad, catalog number: 1610747)
21. 2-mercaptoethanol (Bio-Rad, catalog number: 161-0710)
22. SDS-PAGE running buffer (Bio-Rad, catalog number: 161-0772)
23. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, catalog number: 23225)
24. 1-propanol (Sigma-Aldrich, catalog number: 402893)
25. Ammonium bicarbonate (Sigma-Aldrich, catalog number: A6141)
26. Trypsin, sequencing grade (Promega, Fisher Scientific, catalog number: PRV5111)
27. Formic acid (Sigma-Aldrich, catalog number: 27001)
28. Acetonitrile (Sigma-Aldrich, catalog number: 34851)
29. OptiPrep medium (Progen Biotechnik GmbH, catalog number: 1114542)
30. SDS-PAGE Running Buffer (10× stock; use at 1×) (see Recipes)

Equipment

1. Incubator capable of maintaining 37°C (for example: Fisherbrand, Isotemp Microbiological Incubator)
2. Centrifuge with capacity for 50 ml conical tubes (for example: Beckman Coulter, Avanti JXN-30 using a JA-14.50 rotor)
3. Ultracentrifuge with capacity for 38.5 ml tubes, capable of 129,000 × g (for example: Sorvall WX Ultra 90 centrifuge using AH-629 rotor)
4. NanoSight LM10 (Malvern Panalytical, Worcestershire, UK)
5. ZetaSizer NanoSeries equipped with a HeNe laser source ($\lambda = 633$ nm) and avalanche photodiode (Malvern

- Panalytical, Worcestershire, UK)
- 6. Mini-PROTEIN® Tetra Vertical Electrophoresis Cell (Bio-Rad, catalog number: 1658004)
 - 7. Speed-vac (for example: Thermo Fisher Scientific SC210A SpeedVac Concentrator, catalog number: SC210A-230)
 - 8. Barocycler (Pressure Biosciences Inc., HUB 440-SW16, Easton, MA, US)
 - 9. Orbitrap LC-MS/MS system (for example: Thermo Scientific Orbitrap Fusion Lumos equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific, catalog number: ES071)
 - 10. Autosampler (for example: Thermo Scientific Dionex UltiMate 3000 Rapid Separation Well Plate Autosampler (Thermo Fisher Scientific, catalog number: 5840.0010)
 - 11. Ultra-high performance liquid chromatography (UHPLC) system (for example: Dionex Ultimate 3000 RSLCnano system, Thermo Fisher Scientific, catalog number: ULTIM3000RSLCNANO)

Software

- 1. NTA 2.3 Nanoparticle Tracking and Analysis software (Malvern Panalytical, Worcestershire, UK)
- 2. Dispersion Technology Software (DTS, Malvern Panalytical, Worcestershire, UK) used for dynamic light scattering analysis
- 3. Scaffold version 4.8.2 (Proteome Science Inc., Portland, Oregon, US), used for protein identification after mass spectrometry
- 4. Mascot (version 2.6.1, Matrix Science, London, UK), used for protein identification after mass spectrometry
- 5. X! Tandem (version 1.7.18) used for protein identification after mass spectrometry
- 6. R (<https://cran.r-project.org/>) and relevant R packages: Peptides, Limma, and ggplot2. Used for biochemical analysis of identified proteins, statistical analysis, and generation of volcano plots or other visualizations of the data

Procedure

A. Isolation of membrane vesicles (MVs)

1. Grow bacterial strains
 - a. Streak each strain individually from frozen stocks or stab vials onto MRS agar plates. For anaerobic growth, place plates in an AnaeroJar jar with an AnaeroGen anaerobic gas generation sachet and anaerobic indicator added. Grow at 37°C for approximately 48 h or until individual colonies are visible.
 - b. Inoculate a single bacterial colony into 3 mL MRS broth. Grow overnight at 37°C without shaking in an AnaeroJar as in the previous step.
 - c. Add 1 mL from the primary culture to 50 mL MRS broth in a sterile 250 mL Erlenmeyer flask. Grow overnight as in previous steps.
2. Collect and filter supernatant
 - a. Transfer the overnight culture into a 50 mL conical centrifuge tube and centrifuge at 5,000 × g for 30 min.
 - b. Decant supernatant into a new tube and centrifuge again at 5,000 × g for 30 min.
 - c. Repeat Step A2b once or twice more, until no cell pellet is visible on the sides or bottom of the tube after centrifugation. These additional centrifugation steps are not strictly required but make it easier to filter the supernatant in the following step.
 - d. Pass the supernatant through a 0.45 µm filter. This may be done using a 30 mL syringe and syringe filter or using a vacuum apparatus with an appropriate filter for larger volumes of supernatant.
3. Ultracentrifugation to isolate MVs
 - a. Transfer 36 mL of the filtered supernatant to an ultracentrifuge tube and assemble into the rotor.

- b. Ultracentrifuge at $126,000 \times g$ for 1.5 h at 4°C.
- c. Decant the supernatant (the pellet is often invisible).
- d. Add 1 ml of 1× phosphate-buffered saline (PBS) and incubate overnight at 4°C to resuspend the pellet.

Note: If proceeding for gradient ultracentrifugation, resuspend in 150 μ L of PBS-OptiPrep mixture instead (see Step A4a below).

4. Gradient ultracentrifugation of MVs
 - a. Resuspend MV pellets in 150 μ L of a PBS-OptiPrep mixture (45% v/v) and move the suspension to the bottom of a 5 mL ultracentrifuge tube.
 - b. Make several 0.8 mL PBS-OptiPrep solutions of lower density (e.g., 35%, 30%, 25%, 20%, 15%, and 10%) and sequentially cover the base layer containing MVs with layers of decreasing density. Avoid adding bubbles or disrupting previous layers as you add.
 - c. After all layers have been added, allow the tube to settle for 1 h.
 - d. Ultracentrifuge the gradient at $236,000 \times g$ for 3 h in a Sorvall WX Ultra 90 centrifuge using an AH-650 rotor.
 - e. Following centrifugation, gently sequentially remove and place 10 equal-volume (~500 μ L) fractions into separate tubes.
 - f. Assess the different density fractions for MV concentration, protein composition, and other characteristics using the methods described below.

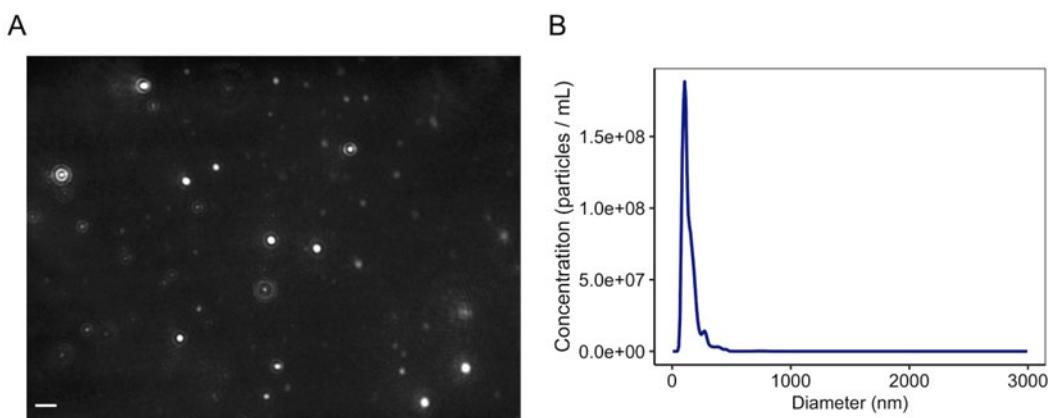


Figure 3. Nanoparticle tracking analysis (NTA) of *Lactobacillus* membrane vesicles.

A. Representative frame taken from a NanoSight LM10 video of *L. acidophilus* MVs. MVs purified from an MRS culture were diluted 1:1,000 in PBS prior to NTA. Scale bar indicates 2 μ m. B. Representative MV size distribution from NTA results, taken from an average obtained from three 60 s videos.

B. MV characterization

1. Nanoparticle tracking analysis (NTA) to determine particle size distribution and concentration
 - a. Dilute MVs in 1× PBS – make several serial dilutions such as 10×, 100×, and 1,000×. Detection of particles by the NTA software is best when there are 20-60 particles within the field of view; therefore, samples need to be diluted, and the optimal dilution for each sample may vary from one isolation to another.
 - b. Using a 1 mL needle-less syringe, add the diluted sample to the NanoSight instrument chamber. Allow a moment for samples to settle and adjust microscope focus and camera settings to optimize clarity of particles in the camera field of view within the NTA 2.3 Nanoparticle Tracking and

- Analysis software. Data can be collected at room temperature.
- c. Capture data as triplicate readings of 60 s exposure at 30 frames per second, and analyze using auto particle detection and tracking parameters: detection threshold, pixel blur, minimum track length, and minimum expected particle size. A representative image of NTA of vesicles from *Lactobacillus acidophilus* ATCC 53544 is shown in Figure 3. As NTA 2.3 does not automatically add scale bars to images, they can be added by converting pixels to μm by dividing pixels by 9.036 (when using a 30 \times objective).
 2. Dynamic light scattering (DLS) to measure MV size distribution
 - a. Dilute MV samples 10-fold in 0.1 \times PBS pH 7.4 and add to disposable DLS cuvettes.
 - b. Carry out DLS analysis using ZetaSizer NanoSeries equipped with a HeNe laser source ($\lambda = 633 \text{ nm}$) and analyze using Dispersion Technology Software. Carry out measurements at 25°C. For each sample, take the average of five runs of 10 s each for the autocorrelation function, and repeat this three to six times.
 3. Zeta Potential measurement to determine MV surface charge
 - a. Dilute MV samples and load into disposable cuvettes as for DLS.
 - b. Measure zeta potential using ZetaSizer NanoSeries equipped with a HeNe laser source ($\lambda = 633 \text{ nm}$) with Dispersion Technology Software. Carry out measurements at 25°C in triplicate for each sample.
 4. SDS-PAGE for qualitative analysis of proteins contained in MVs
 - a. Prepare samples: based on MV concentration as determined by NTA, take equal amounts of each MV sample in $\leq 15 \mu\text{l}$ volume in 1.5 ml centrifuge tubes. Adjust sample volumes to 15 μl using water or 1 \times PBS. (Volumes may be adjusted depending on the well capacity of SDS-PAGE gels to be used.)
 - b. Add 5 μl (or required volume to result in 1 \times final concentration) of 4 \times Laemmli sample loading buffer to each sample, then denature sample proteins by boiling for 5 min, chilling on ice for 5 min, and centrifuging for 15 min at 16,000 \times g in a benchtop centrifuge.
 - c. Assemble a Bio-Rad Mini-PROTEAN Vertical Electrophoresis cell according to the manufacturer's instructions with a 4-15% Mini-PROTEAN® TGX pre-cast protein gel.
 - d. Load denatured MV samples (supernatant only) into the gel and run at 200 V until the dye front reaches the bottom of the gel.
 - e. Remove the gel from its casing and rinse briefly in water; then, stain in GelCode™ Blue Stain Reagent, followed by destaining using water, to visualize MV protein bands. Figure 3 of Dean *et al.* (2019) may be referred to for an example of an SDS-PAGE gel of MVs from three LAB species.
 5. Shotgun Proteomics Analysis to identify protein contents of MVs
 - a. Sample preparation
 - i. Resuspend MV pellets with 10 ml of 10% n-propanol in 50 mM ammonium bicarbonate (ABC) buffer.
 - ii. Normalize sample protein concentration using the Pierce BCA Protein Assay Kit.
 - iii. Digest samples (50 μg) in solution with sequencing-grade modified trypsin at a 1:50 (w/w) enzyme:substrate ratio in a barocycler for 90 min (90 cycles: 50 s on at 45 kpsi, 10 s off).
 - iv. Evaporate digested samples via speed-vac and store at -20°C until analysis by LC-MS/MS.
 - b. LC-MS/MS
 - i. Reconstitute dried samples in 0.1% formic acid in water (solvent A) and load 3 μg digested protein onto a reversed-phase C18 column by a Dionex Ultimate WPS-3000 autosampler connected to an Orbitrap Fusion Lumos equipped with a Nanospray Flex Ion Source in data-dependent acquisition mode.
 - ii. Separate peptides across a 90 min gradient of 2-60% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min using a Dionex Ultimate 3000 RSLCnano system.
 - iii. Save the raw, mgf, and mzid files output for each sample for downstream analysis.
 - c. Protein identification
 - i. Search mass spectrometry data using Mascot and X! Tandem using the appropriate genome

- file for the organism. Validate peptide-spectrum matches with Scaffold.
- ii. Set fragment ion mass tolerance to ± 0.60 Da and parent ion tolerance to ± 0.60 Da. Set proteins identification thresholds to ≥ 2 peptides (protein probability 80%, peptide probability 95%). Export list of identified proteins to CSV.
 - iii. Analyze the output CSV files with R.

Data analysis

Proteomics analysis:

1. Analyze all MS/MS samples using Mascot (Matrix Science, London, UK) and X! Tandem (The GPM, thegpm.org). Have Mascot set up to search the correct database sequence file (generated by protein .fasta input) and a corresponding common contaminants file. Set assumed cleavages to digestion using trypsin.
2. Set up X! Tandem to search a reverse concatenated subset of the correct database sequence file, which only contains proteins identified by Mascot in analyzed samples.
3. Use Scaffold (Proteome Software Inc., Portland, OR, USA) to validate MS/MS based peptide and protein identifications. Set peptide-spectrum match settings to: fragment ion mass tolerance = ± 0.60 Da and the parent ion tolerance = ± 0.60 Da. Set protein identification thresholds to ≥ 2 peptides (protein probability 80%; peptide probability 95%).
4. Analyze proteomics data output to CSV files with R
 - a. Determine physicochemical characteristics of peptides/proteins using the Peptides R package.
 - b. Perform the two-sample t-tests using an empirical Bayes method to adjust the estimate of variance of each protein using the Limma R package, the output of which is each protein with its log₂ fold-change and moderated p-value corresponding to the moderated t-statistic, as previously described (Dean *et al.*, 2020).
 - c. Use the ggplot2 R package to create volcano plots and other visualizations of the data (see Figure 4 for an example volcano plot using proteomics data).

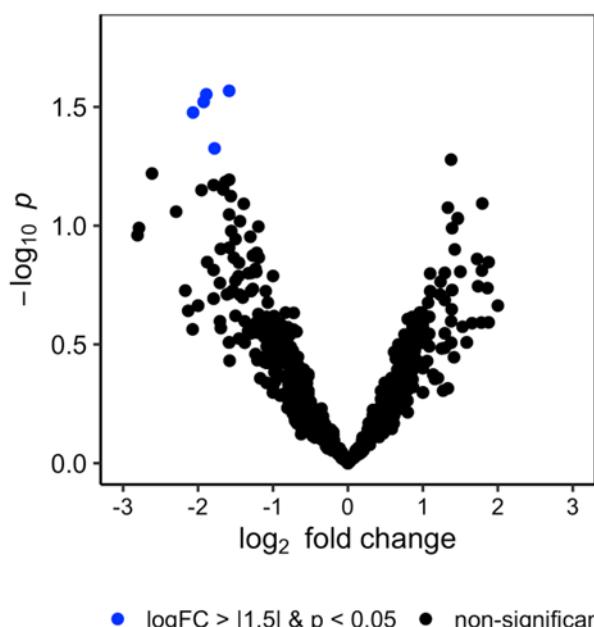


Figure 4. Volcano plot of proteomics performed on *Lactobacillus* membrane vesicles.

Representative volcano plot of \log_2 fold change of proteins identified in *L. acidophilus* MVs treated with inducer relative to an untreated control. Identified protein data in triplicate samples were put through the empirical Bayes method based on the Limma R package and plotted using ggplot2. Proteins with significant

change are colored in blue.

Recipes

1. SDS-PAGE Running Buffer (10× stock; use at 1×)

25 mM Tris base
192 mM glycine
0.1% SDS
Dissolve in water as solvent

Acknowledgments

This protocol is adapted from previous work (Dean *et al.*, 2019 and 2020). Funding for this effort was provided through Core funds of the Naval Research Laboratory (SND, SAW). Additional financial support was provided through the American Society for Engineering Education (ASEE) post-doctoral fellowship program (JCC). The AFM image shown in the graphical abstract was taken by Clarettta Sullivan, Air Force Research Laboratory, Wright Patterson Air Force Base.

Competing interests

The authors declare no competing interests.

Ethics

None of the protocols described here include studies with animals or human subjects.

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Microscopic Detection of ASC Inflammasomes in Bone Marrow Derived Macrophages Post Stimulation

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Abstract

An inflammasome is an intracellular multiprotein complex that plays important roles in host defense and inflammatory responses. Inflammasomes are typically composed of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), cytoplasmic sensor protein, and the effector protein pro-caspase-1. ASC assembly into a protein complex termed ASC speck is a readout for inflammasome activation. Here, we provide a step-by-step protocol for the detection of ASC speck by confocal microscopy in Bone marrow derived macrophages (BMDMs) triggered by chemical stimuli and bacterial pathogens. We also describe the detailed procedure for the generation of BMDMs, stimulating conditions for inflammasome activation, immunofluorescence cell staining of ASC protein, and microscopic examination. Thus far, this method is a simple and reliable manner to visualize and quantify the intracellular localization of ASC speck.

Keywords: ASC, Inflammasome, Fluorescence staining, Confocal microscopy, NLRP3, AIM2, NLRC4

This protocol was validated in: J Clin Invest (2020). DOI: 10.1172/JCI138234

Graphical Abstract:

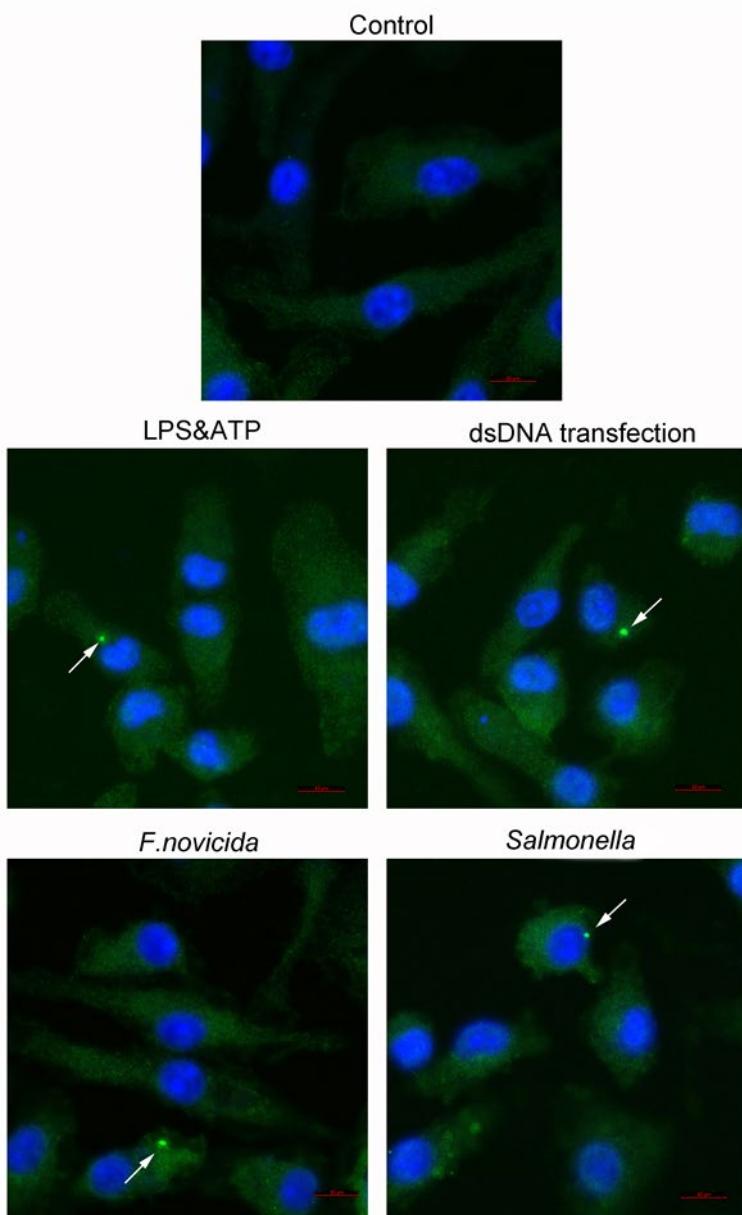


Figure 1. Confocal microscopy detection of ASC speck formation in untreated WT BMDMs and WT BMDMs stimulated with LPS and ATP, transfected with dsDNA, and infected with *F. novicida* or *Salmonella* as indicated.

Arrow indicates the ASC speck. Scale bars: 10 μm .

Background

The innate immune system has a key role in initiating and orchestrating host defense by detecting invading pathogens through membrane-bound and cytosolic pattern recognition receptors (PRRs), which recognize pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs). Inflammasome activation is an

essential innate immune event in response to pathogenic infection and sterile stimuli that causes the initiation of pyroptotic cell death and the release of the proinflammatory cytokines IL-1 β and IL-18. Inflammasome assembly is triggered by the activation of upstream sensors, such as NLRP1, NLRP3, AIM2, NLRC4, and PYRIN. Upon activation, sensor proteins form a complex in an ASC-dependent and -independent manner to mediate caspase-1 cleavage and activation. Cleaved caspase-1 in turn leads to the maturation of proinflammatory cytokines IL-1 β and IL-18 and the process of GSDMD-mediated pyroptosis (Rathinam and Fitzgerald, 2016). The adaptor ASC protein is composed of a PYRIN domain (PYD) and caspase recruitment domain (CARD), which help ASC function as an adaptor to interact with upstream sensor and effector caspase-1 (Agrawal and Jha, 2020). ASC speck formation is a hallmark of inflammasome activation. Confocal microscopy and flow cytometry are two major methods to detect ASC speck formation (Stutz *et al.*, 2013; Sester *et al.*, 2015; Beilharz *et al.*, 2016; Hoss *et al.*, 2018).

We performed and published the determination of ASC speck formation in BMDMs after the activation of NLRP3, AIM2, and NLRC4 inflammasomes (Guo *et al.*, 2020). In comparison with the method using ASC-GFP fusion protein and flow cytometry, this procedure is able to detect endogenous ASC speck formation and can visualize and quantify subcellular localization of the inflammasome complex with the help of cellular organelle staining. This protocol can be utilized to evaluate any ASC speck formation in other cells after ASC-dependent inflammasome activation.

Materials and Reagents

1. Microscope Slides (Citotest, catalog number: 198105)
2. 12-well plate (Jet, catalog number: TCP011012)
3. 10 cm cell culture dish (Jet, catalog number: TCD010100)
4. 15/50 mL sterile centrifuge tube (Jet, catalog number: CFT011500)
5. Serological Pipet (JETBIOFIL, catalog number: GSP-010-005/GSP-010-010)
6. 25 cm Cell Scraper (BIOFIL, catalog number: CSC011025)
7. Microscope Cover Glass (NEST, catalog number: 801008, Φ 15 mm)
8. 5 mL syringe with 26 G needle
9. 20 mL syringe with 18 G needle
10. Mice (C57/BLJ6, Beijing Vital River Laboratory Animal Technology Co., Ltd)
11. RPMI 1640 (Gibco, catalog number: 31800022, 4°C)
12. DMEM/F12 (Gibco, catalog number: 12500062, 4°C)
13. FBS (Hyclone, catalog number: SH30084.03, -20°C/4°C)
14. Nonessential amino acids (Gibco, catalog number: 111140-050, 4°C)
15. Penicillin-streptomycin (Gibco, catalog number: 15140-122, 4°C)
16. PBS (Gibco, catalog number: 21600-069, 4°C)
17. 4% paraformaldehyde (PFA) (Sangon Biotech, catalog number: E672002-0500, 15-25°C (RT))
18. BSA (Sangon Biotech, catalog number: A500023, 4°C)
19. Anti-ASC (AdipoGen, catalog number: AG-25B-0006, -20°C)
20. Saponin (Sigma, catalog number: 47036, 4°C)
21. Fluorescence conjugated secondary antibody (Alexa Fluor™ 488 goat anti-Rabbit) (Invivogen, catalog number: A11008, -4°C)
22. LPS (Invivogen, catalog number: tlr1-smlps, -20°C)
23. ATP (Sigma, catalog number: FLAAS, -20°C)
24. X-fect Transfection Reagent (X-fect polymer, X-fect buffer) (Clontech, catalog number: 631318, -20°C)
25. 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) (CST, catalog number: 8961 S, -20°C)
26. Anti-fluorescence attenuation sealant (Solarbio, catalog number: S2100, 4°C)
27. TWEEN® 20 (Sigma-Aldrich, catalog number: P2287)
28. Cell-neubauer improved (LW Scientific, 0.0025 mm2)
29. BBL™ Trypticase™ Soy Broth (BD, catalog number: 211768, RT)
30. NaCl (Sangon Biotech, catalog number: A501218-0001, RT)
31. Tryptone (OXOID, catalog number: LP0042, RT)

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32. Yeast extract (OXOID, catalog number: LP0021, RT)
33. Agar (SbaseBio, catalog number: A010-1.1, RT)
34. TSB solid media (see Recipes)
35. TSB liquid media (see Recipes)
36. LB solid media (see Recipes)
37. LB liquid media (see Recipes)
38. 1% BSA (see Recipes)
39. 0.1% saponin (see Recipes)
40. 0.1% PBST (see Recipes)

Equipment

1. Ophthalmic scissors (Beijing Bao Yuan Industrial Technology, catalog number: M-Y003)
2. Ophthalmic forceps (Beijing Bao Yuan Industrial Technology, catalog number: M-Y005)
3. Confocal Microscope (ZEISS, model: LSM880)
4. Microscope (ZEISS, model: Primo vert iLED)
5. Biological Safety Cabinets Clean Benches (ThermoFisher Scientific, model: 1300)
6. CO₂ Incubator (ThermoFisher Scientific, model: 3111)
7. Portable Pipet-Aid (Drummond, catalog number: 4-000-201)
8. Diaphragm vacuum pump (Tianjin Jinteng Experiment Equipment, model: GM-0.33A)
9. Nanophotometer p-class (IMPLEN, model: NT-80)
10. -80°C freezer (Thermo, model: FDE30086FV)

Software

1. ZEN black_2-3SP1 (ZEISS, <https://www.zeiss.com/corporate/us/home.html>)
2. ZEN blue 2.6 (ZEISS, <https://www.zeiss.com/corporate/us/home.html>)

Procedure

A. Culture of *Francisella novicida* (U112 strain) and *Salmonella* Typhimurium (SL1344 strain)

1. *Francisella novicida* (U112)
 - a. Recovery of *Francisella novicida*: Streak the frozen *Francisella novicida* stock onto TSB solid media (with 1% L-acetylcysteine) and place in a 37°C incubator for 24 h.
 - b. Culture of *Francisella novicida*: Place a single colony of *Francisella novicida* into 5 ml TSB liquid media (with 1% L-acetylcysteine) and grow in 37°C shaker with 200 rpm rotation overnight.
 - c. Subculture of *Francisella novicida*: Transfer 3 ml of overnight cultured *Francisella novicida* into 6 ml fresh TSB liquid media (with 1% L-acetylcysteine) and incubate at 37°C while shaking at 200 rpm for 2 h.
 - d. Measurement of the concentration of bacteria: Use 1 mL of subcultured *Francisella novicida* to measure the value of OD₆₀₀ with the Nanophotometer p-class (IMPLEN, NT80) and calculate the concentration of bacteria according this equation “1 (OD₆₀₀)=1 × 10⁶ CFU/µL”.
2. *Salmonella* Typhimurium (SL1344)
 - a. Recovery of *Salmonella* Typhimurium: Streak the frozen *Salmonella* Typhimurium stock onto LB solid media and place in a 37°C incubator for 24 h.

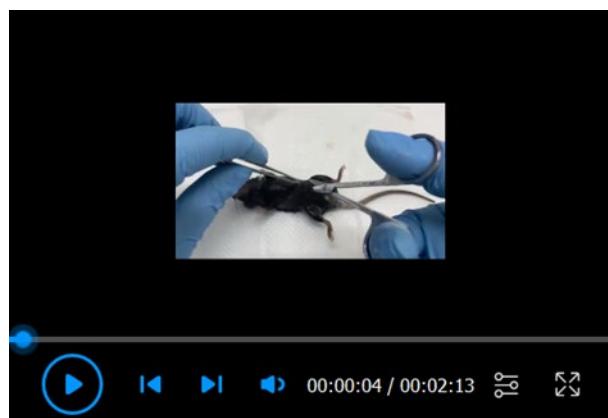
- b. Culture of *Salmonella* Typhimurium: Place a single colony of *Salmonella* Typhimurium into 3 ml LB liquid media and grow in 37°C shaker with 200 rpm rotation overnight.
- c. Subculture of *Salmonella* Typhimurium: Transfer 0.5 mL of overnight cultured *Salmonella* Typhimurium into 2.5 ml fresh LB liquid media and incubate at 37°C while shaking at 200 rpm for 2 h.
- d. Measurement of the concentration of bacteria: Use 1 mL of subcultured *Salmonella* Typhimurium to measure the value of OD₆₀₀ with the Nanophotometer p-class (IMPLEN, NT80) and calculate the concentration of bacteria according to this equation “1 (OD₆₀₀)=1 × 10⁶ CFU/µL”.

B. Preparation of L929 supernatant

1. Seed 5×10^5 L929 cells in 10 cm Petri dish.
2. Culture L929 cells in the incubator (37°C, 5% CO₂) for 7 days in RPMI 1640 media supplemented with 10% FBS.
3. Collect the supernatant with a pipette into a 50 ml centrifuge tube.
4. Centrifuge at 1,962 × g for 10 min and then transfer the supernatant with a pipette into a new 50 mL centrifuge tube. Freeze at -80°C.

C. Generation of BMDMs

1. Sacrifice the mice and spray 75% alcohol on their whole body. Remove the skin and fur from the hind legs and cut off the hind legs after the hip joint. Place in PBS.
2. Remove the muscle tissue from the legs and cut off both ends from the tibia and femur (Video 1).



Video 1. Generation of BMDMs.

(This video was made at the Kunming Institute of Zoology according to guidelines from the Kunming Institute of Zoology on Animal Care and was approved by the Animal Research Ethics Board of the Kunming Institute of Zoology under protocol SMKX-20,6020.)

3. Flush the bone marrow (BM) cells with L929 supernatant-containing DMEM/F-12 media (1:3) supplemented with 10% FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin using a 5 ml syringe with a 26 G needle. Aspirate the flushed bone marrow several times through a 20 mL syringe with an 18 G needle to obtain a single cell suspension.
4. Culture BM cells in the incubator (37°C, 5% CO₂) for 5 days. Use 10 mL media for each Petri dish and add 5 mL of fresh L929 supernatant-containing DMEM/F-12 media to the Petri dish on day 3.

D. Preparation of cell slides

1. Place autoclaved cover slips on a 12-well plate.
2. Wash three times with PBS, for 3 mins each time.
3. Scrape the cells from the culture dish with a cell scraper, resuspend in 10 mL DMEM/F-12 media with 10% FBS, and count cells using a cell counting chamber.
4. Seed 5×10^5 BMDMs into 12-well plates and incubate at 37°C and 5% CO₂ overnight. Make sure the cell confluence the next day is around 60%.

E. Cell stimulation and fixation

1. Change media with 500 μL of fresh DMEM/F-12 media without L929 supernatant before treatment.
2. Treat BMDMs with different conditions, as follows, and incubate at 37°C and 5% CO₂.
 - a. Stimulate BMDMs with 2 μL of LPS (500 ng/ml, 4 h) and 2 μL of ATP (5 mM, 20 min).
 - b. Transfect BMDMs with dsDNA (2 μL of 1.5 μg vector control plasmid) using the X-fect transfection reagent (X-fect polymer 0.45 μL and X-fect buffer 100 μL) for 15 min.
 - c. Infect BMDMs with 10 μL of *Francisella novicida* U112 strain (100 MOI, 12 h).
 - d. Infect BMDMs with 10 μL of *Salmonella Typhimurium* (3 MOI, 1 h).
3. Remove medium by suction from each well and wash three times by gently swirling with PBS.
4. Remove PBS by suction and fix with commercial 4% paraformaldehyde for 15 min at RT.
5. Remove paraformaldehyde by suction and wash the fixed BMDMs on the cover slip by gently swirling with 500 μL PBS three times.

F. Immunofluorescence staining

1. Block and permeabilize the cells in 250-300 μL 1% BSA with 0.1% saponin at 15-25°C (RT) for 1 h.
2. Remove block buffer by suction and gentle swirling of the cells with PBS three times for 3 min each time.
3. Incubate the cells in 300 μL primary antibody solution (anti-ASC in 1% BSA, 0.1% saponin, 1:150 dilution) at 4°C overnight.
4. Wash the cells three times with 500 μL 0.1% PBST on a rocking platform, for 3 min each time.
5. Stain the cells with fluorescence conjugated secondary antibody in PBS (1:300 dilution) at 15-25°C (RT) for 1 h.
6. Wash the cells three times with 500 μL 0.1% PBST on a rocking platform, for 3 min each time.
7. Stain the cells with 250-300 μL DAPI (5 μg/ml, prepared in PBS) at 15-25°C (RT) for 5-10 min.
8. Wash the cells three times with 0.1% PBST on a rocking platform, for 3 min each time.

G. Immobilization of cover slip

1. Remove the cover slip from the 12-well plate with tweezers and air dry.
2. Drop anti-fluorescence attenuation sealant into the cell-containing side.
3. Attach the cell-containing side of the cover slip to microscope slides (25 × 75 mm). Drop sealant onto the edge of the cover slip to bind the cover slip and microscope slide together and prevent sample drying. Avoid air bubbles and air dry for 10 min at 15-25°C (RT).

H. Confocal scanning and image acquisition

1. Scan cells on the ZEISS-LSM880 confocal microscope (100×, the oil-immersion lens). Perform the examination with the ZEISS-LSM880 confocal laser scanning microscope using the following settings: excitation 488 nm and emission 530 nm for ASC speck detection, normal scanning speed, and frame 1024 × 1024. The ASC speck formation was detected and illustrated in Figure 1. Image acquisition was performed with the software ZEN black_2-3SP1.
2. Analyze the data by using the software ZEN blue 2.6 (Protocol in [supplementary material](#)).

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Recipes

1. TSB solid media (1 L)

Reagent	Final concentration	Amount
BBL™ Trypticase™ Soy Broth		30 g
Agar		15 g

Autoclave at 121°C for 15 min.

Add L-acetylcysteine (1% volume ratio) to the autoclaved media when the temperature is around 60°C.

2. TSB liquid media (1 L)

30 g BBL™ Trypticase™ Soy Broth

Autoclave at 121°C for 15 min.

Add L-acetylcysteine (1% volume ratio) to the autoclaved media when the temperature is around 60°C

3. LB solid media (1 L)

Reagent	Amount
NaCl	10 g
Tryptone	10 g
Yeast extract	5 g
Agar	15 g

Autoclave at 121°C for 15 min

4. LB liquid media (1 L)

Reagent	Amount
NaCl	10 g
Tryptone	10 g
Yeast extract	5 g

Autoclave at 121°C for 15 min

5. 1% BSA

Reagent	Amount
BSA	1%
BSA	1 g
PBS	100 mL

6. 0.1% saponin

Reagent	Amount
saponin	0.1%
saponin	0.1 g
PBS	100 mL

7. 0.1% PBST

Cite as: Li, L. et al. (2021). Microscopic Detection of ASC Inflammasomes in Bone Marrow Derived Macrophages Post Stimulation. Bio-protocol 11(17): e4151. DOI: 10.21769/BioProtoc.4151.

Reagent	Amount
PBST	0.1%
TWEEN® 20	1 ml
PBS	1 L

Acknowledgments

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Competing interests

The authors declare no conflict of interest.

Ethics

The present study was approved by the institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences. Animal experiment in this protocol: SMKX-2016020; validity period: January 2017 to January 2022.

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Simple Methods for the Preparation of Colloidal Chitin, Cell Free Supernatant and Estimation of Laminarinase

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Abstract

Colloidal chitin (CC) is a common substrate used in research work involving chitin-active enzymes (chitinases). Cell free supernatant (CFS) is prepared from fermented broth. Preparation of CC and CFS usually involve large amounts of liquid, which must be separated from the solids. This necessitates the use of a large volume centrifugation facility, which may not be accessible to everyone. Filtration is a viable alternative to centrifugation, and several filter elements are described in the literature. Each of those elements has its own set of disadvantages like non-availability, high cost, fragility, and non-reusability. Here we describe the use of lab coat clothing material (LCCM) for the preparation of CC and CFS. For filtration purposes, the LCCM was found to be functional, rugged, reusable, and cost-effective. Also described here is a new method for the estimation of laminarinase using a laminarin infused agarose gel plate. An easily available optical fabric brightener (OFB) was used as a stain for the agarose plate. The laminarin infused agarose plate assay is simple, inexpensive, and was found to be impervious to high amounts of ammonium sulfate (AS) in enzyme precipitates.

Keywords: Colloidal chitin, Laminarin, Laminarinase assay, Glucan, *Streptomyces rimosus*

This protocol was validated in: Sci Rep (2021), DOI: 10.1038/s41598-021-81807-2

Background

Separation of insoluble solids from liquids may be achieved by either centrifugation or filtration. The use of centrifugation for the preparation of colloidal chitin (CC) and cell free supernatant (CFS) has limitations as a large volume of liquids are involved (Hsu and Lockwood, 1975; Mitsutomi *et al.*, 1995). Preparation of CFS from lab-scale and pilot-scale fermented broth is particularly challenging (Sarkar *et al.*, 2010). Thus, a large capacity centrifuge machine (preferably refrigerated) with suitable rotor must be used (*e.g.*, Sorvall BIOS 16). These facilities may not be available to researchers, especially in developing countries. Under these circumstances, filtration can be a viable alternative to centrifugation.

Most of the filter elements described in the literature (*viz.* filter paper, paper coffee filter, Miracloth, cheese cloth, *etc.*) suffer from one or more of the drawbacks listed below (Sandhya *et al.*, 2004; Javaid and Ali, 2011; Murthy and Bleakley, 2012; Nidheesh and Suresh, 2015; Park *et al.*, 2015): (1) Expensive (Miracloth); (2) Fragile (filter paper and paper coffee filter); (3) Non-reusable (filter paper and paper coffee filter); (4) Poor filtration rate (cheese cloth, filter paper, and paper coffee filter) and (5) Not readily available (paper coffee filter and Miracloth).

In this work, a 280 g per square meter (GSM) 65/35 polyester-cotton blend LCCM was used as a filter element. LCCM was found to be perfectly suitable for the preparation of CC. CFS was prepared from *Streptomyces rimosus* AFM-1 using a similar set of materials and is most suitable for organisms exhibiting pellet growth (*viz.* fungi and actinomycetes) (Koteshwara *et al.*, 2021).

Laminarin ($\beta(1\rightarrow3)$ -glucan interspersed with $\beta(1\rightarrow6)$ -branches) is the long-term carbon storage polymer of glucose found in brown algae (Beattie *et al.*, 1961; Graiff *et al.*, 2016). Laminarinases (($1\rightarrow3$)- β -glucan hydrolase) are a type of hemicellulase that act upon and hydrolyze laminarin. They can be classified as either endo-type (EC 3.2.1.39) or exo-type (EC 3.2.1.58) based on the substrate hydrolysis pattern (Wu *et al.*, 2018). The laminarinase enzyme is produced by a wide variety of organisms and plays an important role in natural ecosystems for the recycling of carbon (Graiff *et al.*, 2016). Bacteria usually only transport substrates with a molecular mass of <600Da (Weiss *et al.*, 1991; Grinter and Lithgow, 2020). Consequently, many marine bacteria produce extracellular enzymes like laminarinases to hydrolyze high molecular mass substrates (*e.g.*, laminarin: 2,000-7,000 Da) (Yang *et al.*, 2020). β -Glucan hydrolases are used in the food industry, medicine, and biotechnology (Qin *et al.*, 2017).

Commercial laminarin used in the laboratory is usually sourced from either *Laminaria digitata* (Sigma-Aldrich L9634) or *Eisenia bicyclis* (TCI L0088). Interestingly, laminarin has similarities to the β -glucans found in the fungal cell wall. Thus, it is used to evaluate the anti-fungal β -glucan hydrolases produced by various organisms, especially the biocontrol agents (Scott and Schekman, 1980; Watanabe *et al.*, 1989; De la Cruz *et al.*, 1995; Aktuganov *et al.*, 2008). Under these circumstances, detection and estimation of laminarinase enzyme assumes importance.

AS precipitation continues to be the most preferred method for the separation of proteins from CFS due to the following reasons (Wingfield, 2001; Duong-Ly and Gabelli, 2014): (1) It is inexpensive, simple, germicidal, highly soluble in water, and exerts a stabilizing effect on the precipitated proteins; (2) For the precipitation of native and non-recombinant proteins; (3) It can inhibit proteolytic activity; and (4) Precipitation of recombinant protein without an affinity tag or an unexposed affinity tag.

AS precipitation is a non-specific method. Thus, optimization of the AS concentration must be carried out to reduce the amount of contaminating proteins. AS interferes with most enzyme assays, thus the precipitates must be dialyzed before evaluation. A simple and rapid assay that provides results without the need for dialysis would ease the AS optimization task; this idea was the motivation for this research work (Koteshwara *et al.*, 2021).

Plate assays for enzymes are inherently much simpler than liquid assays. This is due to their simple design, ability to evaluate multiple samples in a single plate, and the fact that they provide direct qualitative/ semi-quantitative results (Teather and Wood, 1982; Samad *et al.*, 1989; Gulati *et al.*, 1997; Meddeb-Mouelhi *et al.*, 2014; Sawant *et al.*, 2015; Patil and Chaudhari, 2017). We have developed a plate assay for laminarinase using a laminarin-infused agarose gel. Laminarin is a translucent substrate necessitating the use of a dye for the detection of zones of hydrolysis. An easily available OFB Tinopal CBS-X (Disodium 4,4'-bis(2-sulfostyryl) biphenyl) was tested as a stain and found suitable. The plate assay provided accurate, reproducible results even in the presence of a high concentration of AS (Koteshwara *et al.*, 2021). Congo red is an extensively used dye for the detection of polysaccharide hydrolysis but is not recommended for use with laminarin gel plate assay due to poor contrast (Wood *et al.*, 1988). Many wide-ranging and complicated methods can be found in the literature for the preparation of CC, CFS, and laminarinase plate assay. Simple alternatives to complex protocols are described here.

Materials and Reagents

1. Glass funnel plain (60° Angle and short stem) (Borosil, catalog number: 6140077)
2. Petri dishes (3.9" and 5.9" diameters) (Borosil, catalog numbers: 3165A77 [3.9"]; 3165081 [5.9"])
3. Conical flask with screw cap (250 mL capacity recommended) (Borosil, catalog number: 5021024)
4. Plastic tray (preferably polypropylene) (Tarsons, catalog number: 240090)
5. Cellulose membrane dialysis tubing (14 kDa cutoff) (Sigma-Aldrich, catalog number: D9652)
6. Steam indicator tape (3M, catalog number: 1322)
7. Dry heat indicator strip (HiMedia Laboratories, catalog number: LA812)
8. Laminarin derived from *Laminaria digitata* (Sigma-Aldrich, catalog number: L9634)
9. Shrimp chitin powder (HiMedia Laboratories, catalog number: RM1356)
10. $(\text{NH}_4)_2\text{SO}_4$ (HiMedia Laboratories, catalog number: GRM1192)
11. KH_2PO_4 (HiMedia Laboratories, catalog number: GRM2951)
12. K_2HPO_4 (HiMedia Laboratories, catalog number: GRM168)
13. Yeast extract powder (HiMedia Laboratories, catalog number: RM027)
14. Malt extract powder (HiMedia Laboratories, catalog number: RM004)
15. Glucose (HiMedia Laboratories, catalog number: GRM016)
16. NaCl (HiMedia Laboratories, catalog number: GRM3954)
17. Agarose (HiMedia Laboratories, catalog number: MB002)
18. $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ (Sisco Research Laboratories, catalog number: 85611)
19. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Sisco Research Laboratories, catalog number: 97868)
20. $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$ (Sisco Research Laboratories, catalog number: 35243)
21. Tinopal CBS-X 99% pure (AksharChem, catalog number: AKSHAR1979)
22. 37% w/w hydrochloric acid (Merck Life Science, catalog number: 1003172011)
23. 65/35 polyester-cotton blend 280 GSM LCCM (generic purchased from local market or collected from an old laboratory coat)
24. Distilled water (prepared in-house)
25. Laminarin infused agarose gel (see Recipes)
26. Tinopal CBS-X OFB staining solution (see Recipes)
27. Chitin acid slurry (see Recipes)
28. CCMS broth medium (see Recipes)

Note: All materials were kept at room temperature, protected from excessive humidity.

Equipment

1. Microwave oven (Samsung, model: MS23F301TAK/TL)
2. Antibiotic Zonescale (HiMedia Laboratories, catalog number: PW297)
3. Glazed porcelain or plastic Buchner funnel with fixed perforated plate 6" diameter (Porcelain: Fisher Scientific, Fisherbrand™, catalog number: FB966J; Plastic: Cole-Parmer, Dynalon™, catalog number: WW-06128-10)
4. Rubber adapter for Buchner funnels (optional for use with vacuum pump) (generic)
5. Vacuum pump (optional) (Toshniwal Instruments (Madras), model: TDC6)
6. Magnetic stirrer and round magnetic stirrer bar with pivot ring 8 × 65 mm (REMI Sales and Engineering Ltd., Magnetic stirrer: model: 1ML; Magnetic stir bar: Tarsons, catalog number: 4115)
7. Autoclave or pressure cooker for sterilization (Autoclave: Osworld, model: JRIC-39; Pressure cooker: TTK Prestige., model: Popular 16L)
8. Glass stirring rod and plastic spatula
9. Plastic beaker 2 L with spout and handle (Sigma-Aldrich, Brand™, catalog number: BR41012)
10. Buchner flask (>2 L capacity recommended) (Sigma-Aldrich, Pyrex™, catalog number: SLW1170/14D)
11. Stage micrometer and eyepiece graticule (Erma Inc., model: ESM11)

12. Microscope (Olympus, model: CX41)
13. Porcelain mortar and pestle (Sigma-Aldrich, CoorsTM, catalog number: Z247499)
14. Water distillation system (Borosil, catalog number: 3361-1.5 L)

Software

1. Java runtime environment (Oracle, <https://www.java.com/en/>)
2. Fiji (ImageJ) (Laboratory for Optical and Computational Instrumentation, University of Wisconsin at Madison, USA. <https://imagej.net/Welcome>)

Procedure

Safety Note: Initial steps in the preparation of CC use concentrated mineral acid (HCl). Wearing eye protection device and gloves and following other safety measures are necessary. Perform the steps involving concentrated acid either in a fume hood or in a well-ventilated area.

A. Preparation of CC and CFS using the LCCM based filtration apparatus

A1. Preparation of CC

1. Weigh 10 g of shrimp chitin powder in a 250 mL glass beaker and mix it with 100 mL of 37% w/w HCl. Mix it slowly but thoroughly using a glass rod. Make sure there are no clumps of chitin powder. A very thick slurry will form, which slowly turns brown.
2. Transfer the beaker into a plastic tray filled with chilled water or ice. This will reduce the heat generated due to the addition of the concentrated acid.
3. Keep mixing the slurry every 5 min with the glass rod for 30 min.
4. The slurry will become noticeably less viscous during the final stages of this process.
5. After the acid hydrolysis step, slowly mix the acid slurry with 2 L of chilled distilled water with concomitant mixing. Use a magnetic stirrer for this step. A white fluffy substance will immediately form. Let this suspension mix for at least 15-20 min. Longer mixing times improve the homogeneity of the suspension.
6. Assemble the LCCM based filtration apparatus as depicted in Figure 1A and Video 1. A single layer of the LCCM is enough to filter the CC. Use rubber bands to tightly hold the LCCM in place. Ensure that most of the surface of the perforated plate of the funnel is in contact with the LCCM. Place the filtration apparatus inside a tray to collect any liquids that may drip from the LCCM due to wicking.
7. Slowly pour the acidic CC suspension into the Buchner funnel. This solution is still considerably acidic at this stage. Exercise caution while handling.
8. A clear filtrate will collect in the Buchner flask, and CC is trapped in the LCCM. A vacuum pump may optionally be used to expedite the process.
9. Decant all of the suspension into the funnel. Allow water to drain from the thick CC paste trapped in the LCCM. This may take 30-45 min without a vacuum pump.
10. Carefully remove the rubber bands from the filtration apparatus and remove the LCCM from the funnel. Keep the LCCM on a flat surface. Scrape and transfer all of the thick CC paste into a 2 L beaker filled with distilled water. Use a magnetic stirrer and mix this suspension for 15-20 min.
11. Wash the LCCM in tap water and dry it if you intend to reuse it. The drying step may be avoided, but in our experience, it considerably improves the filtration rate. Reassemble the filtration apparatus as per step 6 and repeat the filtration process (Steps 7-11).
12. This process must be repeated until the pH of the CC slurry is near neutral. Usually, 3-5 repetitions of the filtration process are required to get near neutral cake. The number of repetitions can be reduced by allowing more time for the water to drain from CC paste (Step 9). Each filtration cycle takes about 2 h

to complete.

13. Collect the CC paste and sterilize it in an autoclave or a pressure cooker at 121°C for 15min. Use steam indicator tape to validate the sterilization process.
14. Store the sterilized CC at 4°C until use in a tightly closed container. Under this condition, the product can be stored for at least 6 months.

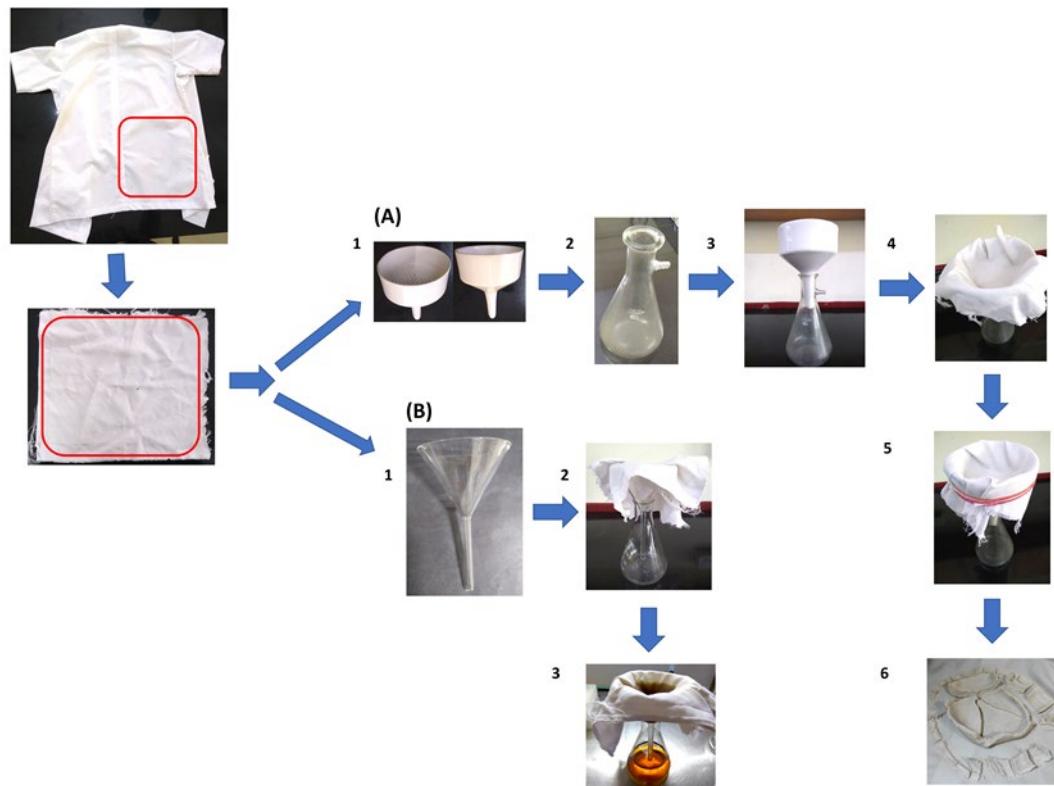
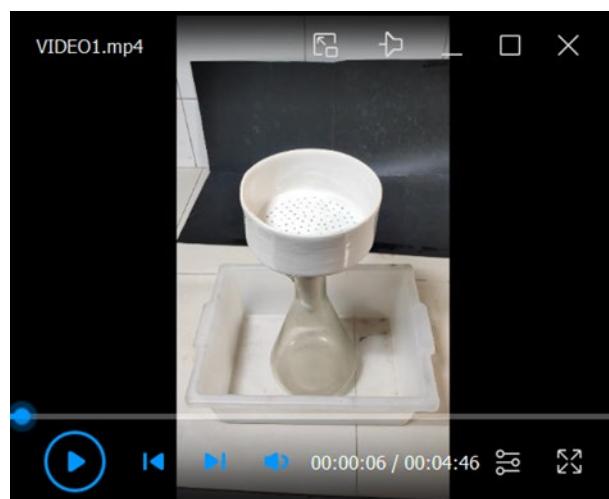


Figure 1. Laboratory coat clothing material (LCCM) based filtration apparatus.

(A) Preparation of colloidal chitin (CC). (B) Cell free supernatant (CFS). A piece of LCCM is taken from an old laboratory coat.



Video 1. Assembling the LCCM based filtration apparatus and preparation of CC

A2. Preparation of CFS

1. This procedure is best suited for fungi and actinomycetes that exhibit pellet growth.
2. Sterilize the LCCM and conical flask cap in an autoclave or pressure cooker at 121°C for 15 min. Sterilize a conical flask and a glass funnel in a hot air oven at 170°C for 45 min. Use appropriate indicator tapes/strips to validate the sterilization process.
3. Assemble the CFS filtration apparatus as depicted in Figure 1B under sterile conditions inside a laminar flow chamber. Use sterile tweezers to push the LCCM into the funnel.
4. The use of rubber bands for securing the LCCM to the funnel is not required.
5. A liquid culture of *Streptomyces rimosus* AFM-1 grown in colloidal chitin mineral salts medium (CCMS) was used in this study. The culture exhibited pellet growth. Pour the contents of the fermented broth into the funnel wrapped in LCCM. Wait for the liquid in the funnel to filter into the flask before topping it up.
6. Close the glass flask with the cap and store at 4 °C until further use.
7. Either collect or dispose of the cell mass as per requirement. Decontaminate the cell mass and LCCM before disposal or cleaning, respectively.

B. Extraction and plate assay of laminarinase from *Streptomyces rimosus* AFM-1

B1. AS precipitation

1. Use powdered AS if available, or else grind the granules to a fine consistency using a mortar and pestle. Powdered AS is easy to dissolve in the CFS.
2. Mixing of AS with CFS should be performed at 4°C to avoid denaturation of proteins. Use crushed ice if cold room facility is not available.
3. Weigh the required amount of powdered AS. Add small portions to the CFS. Use a magnetic stirrer at low speed for mixing. Ensure that the previously added portion is completely dissolved before adding the next. High mixing speed or large portions of AS cause frothing and concomitant loss of proteins.
4. Continue stirring the CFS at cold temperature for at least 30 min.
5. Centrifuge at 4,032 × g for 30 min to pellet the precipitated proteins. The centrifugation parameters must be optimized for different proteins.
6. Decant the supernatant and dissolve the protein pellet in a small volume of 50 mM potassium phosphate buffer (pH 6.5) or any other buffer system of your choice. The enzyme content of this solution can be directly analyzed by laminarin-infused agarose plate assay.
7. Similar precipitation experiments can be performed with different concentrations of AS (viz. 30-90% saturation). Such experiments can be used to optimize the AS saturation needed for precipitation while simultaneously reducing the levels of contaminating proteins (Figure 2).

B2. Testing enzyme solutions using laminarin infused agarose plate with OFB staining

1. Prepare a solution of 0.8% agarose and 0.15% laminarin in 50 mM potassium phosphate buffer (pH 6.5) by melting the solution in a microwave oven and pour into a clean Petri dish. Wait for the gel to solidify.
2. Using a cork borer, cut 6 mm diameter wells in the agarose gel.
3. Pour 20-50 µl of laminarinase enzyme or AS precipitated samples into the wells. Use the same volume of either plain buffer or heat-inactivated enzyme solution as a negative control. Refer to the notes section for further instructions.
4. Place the Petri dish in an incubator set at 37°C. Standardize incubation time and temperature for your application before running the full-scale experiments.
5. Meanwhile, prepare a 0.15% solution of OFB Tinopal CBS-X in distilled water. Prepare this solution fresh in a container protected from light.
6. After the incubation time of 4 h, flood the surface of the agarose gel with the OFB solution. Incubate the plate for 15 min in the dark. Discard the OFB solution and destain once with distilled water for 20 min.
7. Observe the Petri dish under 365 nm UV light (Figure 2A and 2B). An antibiotic zonescale or a calibrated scale ruler is recommended for measuring the zones of hydrolysis.

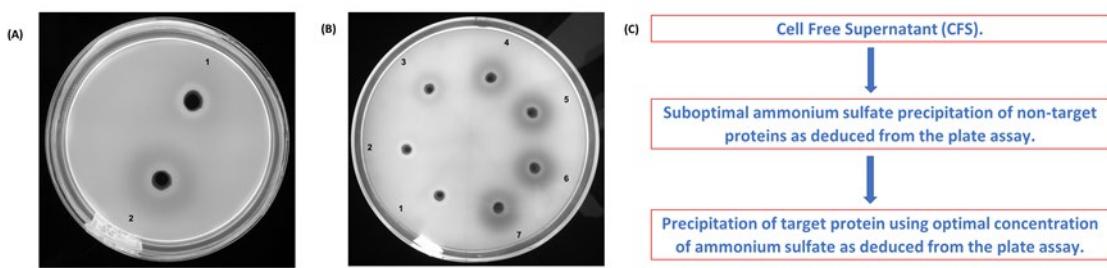


Figure 2. Optimizing the ammonium sulfate (AS) concentration based on laminarin infused agarose plate assay.

(A) Heat denatured (1) and native (2) samples of AS precipitated laminarinase. (B) Enzyme precipitates (30-90% AS treatment). 30% (1); 40% (2); 50% (3); 60% (4); 70% (5); 80% (6) and 90% (7). (C) A suggestive precipitation protocol for improved selectivity.

C. Determination of average Feret's pore diameter of LCCM (see Video 2)

1. Place the graticule in the eyepiece of a microscope as per the manufacturer's recommendation.
2. Place the stage micrometer in the microscope and focus using 4× objective.
3. Align the markings on micrometer and the graticule. Take a picture (P1). This picture will be used for 'define scale' function of ImageJ software.
4. Remove the stage micrometer and carefully place a piece of LCCM without disturbing the focus settings.
5. Nudge the LCCM to bring it into sharp focus. Take a picture with the LCCM in focus (P2). This picture will be used to measure the average Feret's pore diameter in the LCCM.
6. Download the latest Java runtime environment (JRE) and install.
7. Download Fiji software and unpack the zip file (Rueden *et al.*, 2017). This software is provided as a portable application and hence installing is not necessary.
8. Double click on the ImageJ application file located inside the unpacked file folder.
9. The 'Analyze' menu is important for this work. Most of the functions used here are under this menu (viz. 'Set scale' and 'Measure').

Open P1 in ImageJ. Select straight line tool. Draw a line from the left-hand end of the micrometer markings to where the lines on the stage micrometer and graticule align with each other (Figure 3A).

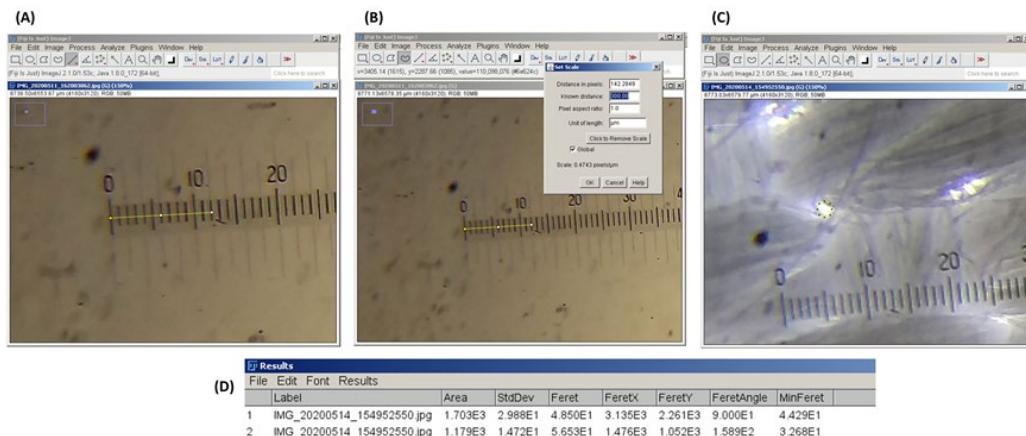


Figure 3. Use of ImageJ for the determination of Feret's average pore diameter.

(A) Drawing a straight line in ImageJ. Stage micrometer and eyepiece graticule are aligned with each other. (B) Setting the measurement scale ('set scale' function). Count the number of markings on the stage micrometer that align with the markings on the eyepiece graticule. (C) Selecting a pore in LCCM

using the oval selection tool. (D) The ‘Results’ window of ImageJ. Note the dimensional parameters of the selected area.

10. Note the number of markings in the stage micrometer that align with the graticule markings. For example, in Figure 3A, it is at the third marking of stage micrometer, which is equivalent to 300 μm .
11. Open the ‘set scale’ option and input 300 in the ‘known distance’ menu. Type ‘um’ in the ‘unit of length’ menu. ImageJ will recognize this as μm . Click the ‘global’ check box and press OK (Figure 3B).
12. ImageJ is now calibrated to perform measurement operations. Open P2 without exiting the software.
13. Select the oval or freehand selection tool. Select a pore in P2 (Figure 3C).
14. Click on ‘measure’ option. A new ‘Results’ window will open, which includes all the dimensional parameters of the selected area in P2 (Figure 3D).
15. Note down the Feret’s diameter.
16. Select another pore and repeat Steps 14-16. Analyze as many pores in P2 per requirement and tabulate the results. Calculate the average value. This will be the average Feret’s pore diameter of LCCM.



Video 2. Determination of average Feret’s pore diameter using ImageJ tool

Data analysis

A. Testing enzyme solutions using laminarin infused agarose plate with OFB staining

1. The zones of hydrolysis can be measured using scale ruler or antibiotic zonescale.
2. Duplicate samples can be included to enhance precision.
3. Calculate the average and, if required, the standard deviation of zone diameters.
4. No special mathematical/statistical tools are needed for this task.
5. Refer to Koteshwara *et al.* (2021) (supplementary material) for more information.

B. Determination of average Feret’s pore diameter of LCCM

1. Use at least three different LCCM pictures for pore size determination.
2. Tabulate the Feret’s diameter values from the ImageJ software.
3. Find the average of Feret’s diameter of pores. A graph denoting the distribution of pore sizes can also be prepared.
4. Refer to Koteshwara *et al.* (2021) (supplementary material) for more information and graph.

5. No special mathematical/statistical tools are needed for this task.

Notes

1. For the laminarin plate assay, run a few trial experiments if the concentration of reagents or buffer systems are changed from what is described here.
2. Cost-effective alternatives are available for most of the items listed under reagents and equipment.
3. The laminarin infused agarose gel method with OFB staining described here can be used to optimize the AS concentration and precipitation time (see Koteswara *et al.*, 2021) for more information) without dialyzing the sample. Data from the optimization experiment can be used to reduce the amount of contaminating proteins (Figure 2B and 2C).
4. Buffer system and laminarin concentration can be changed based on the requirements. Vary the volume of the laminarin-infused agarose gel based on the diameter of the Petri dish used to get the required gel thickness. We recommend adding 25 mL and 65 mL, respectively, for 3.9" and 5.9" diameter Petri dishes.
5. Use sodium azide to inhibit microbial growth in the laminarin-infused agarose gel (see Recipes).
6. Boiling enzyme samples at 100°C for 20 min usually denatures most proteins. Run an experiment to confirm inactivation. Denatured enzyme samples can be used as a negative control.
7. A vacuum pump may be used to speed up CC filtration.
8. Pressure cookers are most convenient for sterilizing small batches of articles. We have been successfully using a 16 L pressure cooker with liquified petroleum gas (LPG) stove for the past several years.
9. There may be other methods for the determination of the average Feret's pore diameter using ImageJ, but the procedure described here is direct and simple.
10. The methods described here are simple, inexpensive, and easy to perform in most of the research laboratories without a large capacity centrifuge for the preparation of CC and CFS. Dialysis, a time-consuming procedure followed commonly during optimization of AS concentration and precipitation time, can be avoided by using the laminarin plate assay described here.

Recipes

1. Laminarin infused agarose gel

50 mM potassium phosphate buffer
0.15% laminarin
0.8% agarose
0.05% sodium azide (optional)
pH 6.5

Dissolve 0.294 g of K₂HPO₄, 0.451 g of KH₂PO₄, 0.15 g of laminarin, 0.05 g of sodium azide (optional), and 0.80 g of agarose in 100 mL distilled water. Heat to dissolve and pour into Petri dish.

2. Tinopal CBS-X OFB staining solution

0.15% Tinopal CBS-X 99% pure
Dissolve 0.15 g OFB in 100 mL distilled water.

3. Chitin acid slurry

10 g shrimp chitin
37%w/w HCl
Slowly mix 10 g chitin in 100 mL of HCl. Proceed as per section A1.

4. CCMS broth medium

Dissolve 2.803 g K₂HPO₄, 1.893 g KH₂PO₄, 1.5 g NaCl, 3 g yeast extract, 0.5 g MgSO₄·5H₂O, 0.01 g FeSO₄·7H₂O, 0.001 g ZnSO₄, 25 g moist CC in 1,000 ml of distilled water. Adjust to pH 7.0.

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Competing interests

The authors declare no competing interests.

Ethics

No human subjects or animals were used for this research work.

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Optimised Method for the Production and Titration of Lentiviral Vectors Pseudotyped with the SARS-CoV-2 Spike

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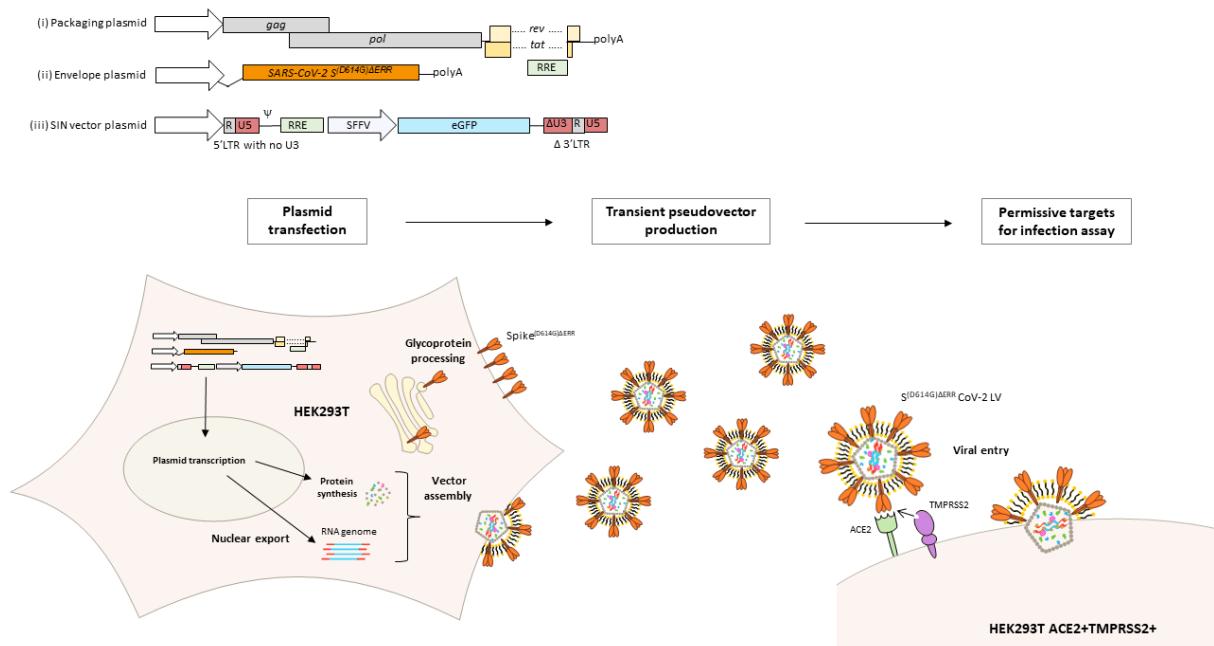
Abstract

The use of recombinant lentivirus pseudotyped with the coronavirus Spike protein of SARS-CoV-2 would circumvent the requirement of biosafety-level 3 (BSL-3) containment facilities for the handling of SARS-CoV-2 viruses. Herein, we describe a fast and reliable protocol for the transient production of lentiviruses pseudotyped with SARS-CoV-2 Spike (CoV-2 S) proteins and green fluorescent protein (GFP) reporters. The virus titer is determined by the GFP reporter (fluorescent) expression with a flow cytometer. High titers ($>1.00 \times 10^6$ infectious units/ml) are produced using codon-optimized CoV-2 S, harbouring the prevalent D614G mutation and lacking its ER retention signal. Enhanced and consistent cell entry is achieved by using permissive HEK293T/17 cells that were genetically engineered to stably express the SARS-CoV-2 human receptor ACE2 along with the cell surface protease TMPRSS2 required for efficient fusion. For the widespread use of this protocol, its reagents have been made publicly available.

Keywords: SARS-CoV-2 glycoprotein, Lentiviral vector pseudotyping, Spike ER retention signal, D614G mutation, Stable ACE2/TMPRSS2 HEK293T/17 cells

This protocol was validated in: J Virol (2021), DOI: 10.1128/JVI.00685-21

Graphical Abstract:



Production and quantification of lentiviral vectors pseudotyped with the SARS-CoV-2 Spike glycoprotein

Background

Studies on SARS-CoV-2 viruses are hampered by the difficulty to produce and manipulate the live viruses that require biosafety level 3 (BSL-3) labs. An alternative to using live virus is to use recombinant lentivirus pseudotyped with the SARS-CoV-2 Spike protein. Pseudotyped viral vectors are very powerful tools for studying biological processes related to enveloped viruses, such as viral entry and immunological response. Pseudotyped viral particles consist of the envelope glycoprotein of one virus with a replication-deficient core of another virus. This allows the deficient core to be dependent on the pseudotyping envelope for target cell entry, thus allowing the investigation of SARS-CoV-2 infection and related serological responses.

Spike is a type-I fusion transmembrane protein expressed on the surface of viral particles as a crown-shaped trimer of heterodimers. In host cells, precursor glycoproteins are proteolytically cleaved by furin at the multibasic S1/S2 site, resulting in dimers composed of an extracellular subunit (S1) containing the receptor-binding domain, which is non-covalently attached to a transmembrane subunit (S2) responsible for viral fusion and subsequent cell entry (Hoffmann *et al.*, 2020a; Peacock *et al.*, 2020). Spike is both necessary and sufficient to induce membrane fusion and cell entry by first binding to its human receptor, ACE2 (hACE2), followed by its proteolytic cleavage by target cell proteases such as the transmembrane protease serine 2 (TMPRSS2) (Hoffmann *et al.*, 2020b; Walls *et al.*, 2020). Thus, Spike has been shown to be the primary target for neutralizing antibodies in COVID-19 convalescent patient sera (Chen *et al.*, 2020; Ju *et al.*, 2020; Pinto *et al.*, 2020).

Spike from different coronaviruses have been successfully pseudotyped on different non-replication competent viruses (Carnell *et al.*, 2017; Grehan *et al.*, 2015; Yan *et al.*, 2007; Fukushi *et al.*, 2005). Accordingly, different viral vectors have been pseudotyped with Spike from SARS-CoV-2, including VSV (Letko *et al.*, 2020; Nie *et al.*, 2020), HIV (Ou *et al.*, 2020; Wu, 2020), and MLV (Pinto *et al.*, 2020; Quinlan *et al.*, 2020) based vectors for neutralization assays, which have been reported to correlate with the live strain. However, a detailed protocol of pseudovector production has not been described for widespread application.

Herein, we describe a fast and reliable protocol for the production of a self-inactivating lentiviral vector

pseudotyped with SARS-CoV-2's Spike glycoprotein and expressing enhanced green fluorescent protein (eGFP) as a marker of infection, which has recently been used to determine neutralisation efficiency of COVID-19 therapeutics on four SARS-CoV-2 variants (Ferrari *et al.*, 2021). The protocol employs a three-plasmid transfection in HEK293T/17 cells with the following plasmids: (i) plasmid expressing the HIV-1 lentiviral genes *gag*, *pol*, *rev*, and *tat*; (ii) a self-inactivating transfer vector encoding eGFP under an internal viral promoter derived from the spleen focus-forming virus (SFFV); and (iii) a plasmid encoding codon-optimized Spike, with or without the prevalent D614G mutation (Korber *et al.*, 2020), under the cytomegalovirus (CMV) promoter with its ER retention signal (amino acid residues 1255 to 1273) deleted as it has been shown to enhance surface expression (Ou *et al.*, 2020). Produced vectors are then quantified by an infectivity assay on genetically engineered HEK293T/17 cells that stably express hACE2 and TMPRSS2 (Supplementary Figure 1). This protocol has been recently used to produce lentiviral vectors pseudotyped with different SARS-CoV-2's Spike variants. These cells have been deposited in the repository of the National Institute for Biological Standards and Control's Covid-19-related research reagents (CFAR #101008) for widespread use.

Materials and Reagents

1. Filtered Pipette tips 1-10, 1-20, 1-200, 100-1,000 μL (Starlab, catalog numbers: S1120-3810 [P10]; S1120-1810 [P20]; S1120-8810 [P200]; S1122-1830 [P1000]) or equivalent
2. 10 cm plates (tissue-culture treated 100 mm dish; Corning, catalog number: 430167) or equivalent
3. 24-well plates (TC-treated 24-well plate; Corning® Costar®, catalog number: 3527) or equivalent
4. Microcentrifuge tube with screw caps write-on graduated with lid latch 1.5 mL
5. 15 mL conical centrifuge tubes (Merck, Corning®, catalog number: CLS430791) or equivalent
6. Nunc™ EasYFlask™ 75 cm^2 cell culture flasks (ThermoFisher, catalog number: 156472) or equivalent
7. Sterile syringes (20 mL), generic
8. Millex-HV syringe filter unit, 0.45 μm , PVDF, 33 mm, gamma sterilized (Merck, catalog number: SLHV033RB) or equivalent
9. 96-well V-bottom plate (96 Well TC-treated microplates, Corning, catalog number: 3894) or equivalent
10. Dry ice
11. Ethanol absolute $\geq 99.8\%$ (Avantor, VWR Chemicals, catalog number: 20821.467DP) or equivalent
12. HEK 293T/17 cells (ATCC, catalog number: CRL-11268)
13. Engineered HEK 293T/17 cells with human ACE2 and TMPRSS2 (NIBSC code CFAR#101008)
14. Plasmids:
 - a. Transfer vector: pCCL.SFFV.eGFP
 - b. Lentiviral transfer vector encoding eGFP downstream of an SFFV promoter in a pCCL backbone.
 - c. Second-generation lentiviral packaging plasmid: pCMVR8.74 (Addgene, catalog number: 22036)
 - d. Glycoprotein expression plasmid: pCDNA-SARS-CoV-2-S^{(D614G)ΔERR}
 - e. SARS-CoV-2 Spike glycoprotein downstream a CMV promoter in a pCDNA backbone. The glycoprotein contains the D614G substitution and deletion of the last 19 amino acids on its carboxyl-terminus to remove its ER retention signal (Δ ERR).
15. Iscove's Modified Dulbecco's Medium (Merck, Sigma-Aldrich, catalog number: I3390) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) (Biosera, catalog number: FB-1001/500)
16. Puromycin (InvivoGen, catalog number: ant-pr-1) or equivalent
17. Reduced Serum media Opti-MEM™ (Thermo Fisher Scientific, Gibco™, catalog number: 31985047)
18. GeneJuice® Transfection Reagent (Merck Millipore, catalog number: 70967)
19. Phosphate-buffered saline (PBS) (Sigma, catalog number: D8537) or equivalent
20. Cell dissociation buffer Enzyme-free PBS-based (Thermo Fisher Scientific, Gibco™, catalog number: 13151014) to avoid surface cleavage of both hACE2 and TMPRSS2
21. Polybrene transfection reagent 10 mg/mL (Merk, catalog number: TR-1003-G)
22. Viability dye (Thermo Fisher Scientific, eBioscience™ Fixable Viability Dye eFluor™ 780, catalog number: 65086514) or equivalent

Equipment

1. Class II biosafety cabinet (Thermo Fisher Scientific, Thermo Scientific™, model: HeraSafe™ 2030i)
2. Water bath (Avantor, VWB2 series, VWB2 2, catalog number: 462-0554) or equivalent
3. Incubator (Eppendorf, model: CellXpert® C170) or equivalent
4. Pipettes (Eppendorf Research® plus, P2, P20, P200, and P1000) or equivalent
5. Cell counter (Chemometec, Automated cell Analyzer NucleoCounter® NC-250™) with Solution 18 AO.DAPI (Chemometec, catalog number: 910-3018) or equivalent
6. Plate centrifuge (Thermo Fisher Scientific, model: Heraeus Multifuge X3R) or equivalent
7. Flow cytometer equipped with a blue and red laser

This protocol utilized a BD LSRLFortessa™ X-20 cell analyser but is not restricted to this equipment. In the absence of a flow cytometer, a fluorescent microscope fitted with a 488 nm excitation laser can be used.

Software

1. FlowJo™ Software, version 10.7.1 (BD Bioscience)
An alternative software for Flow Cytometry analysis can be used.

Procedure

A. Spike LV production by transient transfection

Day 1: Cell seeding

Seed 2.0×10^6 cells HEK293T/17 cells per 10 cm plate in 10 mL of culturing media supplemented with 2 mM L-glutamine and 10% FCS aiming for 80% confluence on day of transfection.

Day 3: Transfection

1. Prepare two sterile 1.5 mL microcentrifuge tubes labeled Tube 1 and Tube 2.
2. Add 5.56 µg of pCMVR8.74, 2.77 µg of pCDNA-SARS-CoV-2-S^{(D614G)ΔERR}, and 4.17 µg of pCCL.SFFV.eGFP, for a total of 12.5 µg of DNA, to Tube 1 (equivalent to 2:1:1.5 plasmid ratios for scalable production).
3. Prepare the transfection mixture in Tube 2, as shown in Table .

Table 1. Transient transfection mixture.

Reagents	µL/10 cm plate
Gene Juice	30
OptiMEM®	470

4. Mix GeneJuice/OptiMEM® transfection mixture using a pipette and incubate for 5 min at room temperature.
5. Pipette 500 µL of transfection mixture into tube containing the plasmid constructs (Tube 1) and gently mix solution five times using the same pipette, avoiding the formation bubbles.
6. Incubate solution for 15 min at room temperature.
7. Pipette the DNA/GeneJuice/OptiMEM solution onto HEK293T/17 cells in a dropwise manner throughout the total surface of the plate.
8. Place plate in an incubator at 37°C with 5% CO₂ for 16-18 h.

Day 4: Media change

1. Carefully remove the culture media from plate 16-18 h post-transfection.
2. Slowly add 7 mL of fresh culturing media supplemented with 2 mM L-glutamine and 10% FCS by pipetting media to one side of the plate to avoid detachment of cells.
3. Place plate back in the incubator at 37°C with 5% CO₂ for 16-18 h.

Day 5: Viral supernatant harvesting

1. Harvest the viral supernatant containing the lentiviral vector pseudotyped with SARS-CoV-2-S^{(D614G)ΔERR} into a 15 mL Falcon previously chilled by either placing it in the fridge or on wet ice for 10 min.
2. Centrifuge at 400 × g for 5 min to remove cellular debris.
3. Clarify viral supernatant with 0.45 µm microfiltration to remove viral aggregates.
4. To avoid multiple freeze-thaw cycles, aliquot viral supernatants into 0.2-0.5 mL aliquots in sterile 1.5 mL microcentrifuge tubes with screw caps.
5. For later use, snap-freeze vials using dry ice and ethanol before storing them at -80°C.
6. For immediate use, store vials on ice until needed.

B. Spike-LV titration by infectivity assay

Day 1:

1. Seeding permissive cells in a 24-well plate:
 - a. Pre-warm IMDM containing 10% FCS, 1% GlutaMAX, and 1 µg/mL Puromycin. This will be referred to as culturing media.
 - b. Harvest stable HEK293T/17-hACE2+TMPRSS2+ cells previously thawed and cultured for at least two passages (with the addition of 1 µg/ml Puromycin from the first passage after thawing) (Figure S1) as follows:
 - i. Remove and discard cellular supernatant.
 - ii. Gently wash cells by pipetting 5 ml of PBS to remove any excess culturing media.
 - iii. Add 3 ml of Cell dissociation buffer Enzyme-free PBS-based to the flask and incubate for 5 min.
 - iv. Gently tap the sides of the flask to prompt cell detachment.
 - v. Add 7 ml of culturing media to flask, collect cells, and pipette into a new Falcon.
 - vi. Centrifuge at 400 × g for 5 min.
 - vii. Discard supernatant and flick Falcon with finger to gently dislodge cellular pellet.
 - viii. Resuspend the cells by adding 5 ml of culturing media.
 - ix. Determine cell density:
 - 1) Passage cells by plating 1:6-1:8 of resuspended cells in a new T75 flask with 9 ml of culturing media, with a recommended minimum seeding of 3.3 × 10⁴ cells/cm².
 - 2) Proceed to Step 1c for titration cell seeding.
 - c. Determine cell density using an Automated cell Analyzer NucleoCounter® NC-250™.
 - d. Resuspend cells at 5.0 × 10⁴ cells/mL in 8 ml per viral vector supernatant to be quantified by titration.
 - e. Add 0.88 µL/mL of polybrene to resuspended cells; the final volume in each well will be 1.1 mL, leading to a final concentration of 8 µg/ml of polybrene.
 - f. Mix the resuspended cells and plate 1 mL/well in a total of seven wells (six wells for vector infection and one well for a no-vector control).
 - g. Add 1 mL of PBS to remaining unoccupied wells.
 2. Preparing Spike LV dilutions for infection of permissive cells:
 - a. If using frozen Spike-LV, thaw in a water bath set at 37°C and immediately use. If using fresh Spike-LV placed on ice, mix viral vectors by pipetting a few times, avoiding the formation of bubbles.
 - b. In a V-bottom 96-well plate, prepare the following 1:4 serial dilutions of Spike-LV in pre-chilled culture media for six points:
 - i. Pipette 200 µL of Spike-LV into one well in the V-bottom plate (e.g., well A1).
 - ii. Pipette 150 µL of pre-chilled complete IMDM into five every other well in V-bottom plate (e.g.,

wells A3, A5, A7, A9, and A11).

Tip: Using every other well on the plate allows moving between 96-well plates and 24-well plates using a multichannel pipette (see Figure 1).

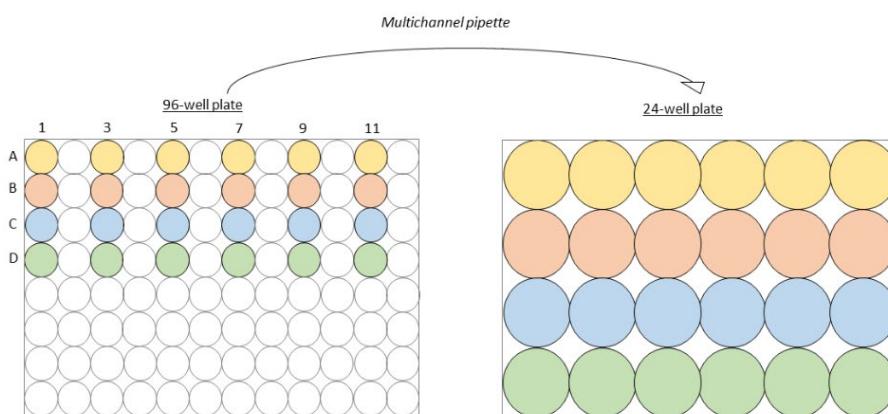


Figure 1. Transfer between 96-well and 24-well plates

- c. Move 50 μL from the first well (A1) containing Spike-LV into the subsequent well (A3).
- d. Gently mix with a pipette ten times, being careful to avoid the formation of bubbles.
- e. Move onto the third well (A5) and continue the serial dilution until reaching the 6th well (A11).
- f. Using a multichannel with every other tip occupied, pipette 100 μL of vector dilutions to each well of HEK293T/17-hACE2+TMPRSS2+ cells already plated in a 24-well plate (Figure 1).
- g. Centrifuge the plate at 1,000 $\times g$ for 20 min to enhance transduction by spinoculation.
- h. Place plate back in the incubator at 37 °C with 5% CO₂ for 72 h.

Day 3: Determining transduction efficiency and titer calculation

1. Discard culture media by flicking 24-well plate into a laboratory box that can be subsequently disinfected or a laboratory sink if the correct risk assessments are in place. Blot the plate on paper to remove excess liquid.
2. Add 200 μL /well of cell dissociation buffer and incubate for 10 min.
3. Gently tap the plate to detach cells.
4. Add 400 μL of PBS/well, resuspend cells by pipetting, and harvest 200 μL into a new V-bottom 96-well plate.

Tip: Using every other well on the plate allows moving between 96-well plates and 24-well plates using a multichannel pipette (Figure 1).

5. Centrifuge the plate at 400 $\times g$ for 5 min.
6. Discard supernatant by quickly flicking plate in an appropriate manner (see step 1) and blot on paper to remove excess liquid.
7. Add 250 μL PBS/well and centrifuge plate at 400 $\times g$ for 5 min.
8. In the meantime, prepare a master mix for Fixable Viability Dye eFluor™ 780 (0.1 μL + 99.9 μL PBS/well).
9. Discard supernatant by quickly flicking plate in an appropriate manner (see step 1) and blot on paper to remove excess liquid.
10. Resuspend cells in 100 μL /well staining master mix.
11. Incubate at room temperature for 30 min in the dark.
12. Add 150 μL PBS/well and centrifuge plate at 400 $\times g$ for 5 min.
13. Discard PBS, blot plate, and resuspend cells in 50 μL of PBS using P200 tips.

14. Acquire plate on flow cytometer with the following bandpass filters:
 - a. RL2 780/60 for Fixable Viability Dye eFluor™ 780.
 - b. BL1 530/60 for fluorescent marker of transduction, eGFP.

Note: If the user is unfamiliar with flow cytometric analysis of cells, it is advisable that a trained flow cytometry operator assists from this step onward.

Data analysis

1. FlowJo analysis

The gating strategy to identify the percentage of eGFP-positive population is depicted in **Figure 2A**, starting with the live cell gate, followed by the forward versus side scatter gate, and the singlets identification. Singlets are then plotted as histograms to identify the percentage of eGFP-positive cells (**Figure 2B**).

2. Determine the percent of transduced cells in each well

When calculating titers, only transduction efficiencies of less than 20% of transduced cells should be considered. This method assumes one integration per cell. Using >20% risks counting cells with multiple integration sites which leads to an underestimation of the titer. Transduction efficiencies between 0.5% and 20% were used to compare vector production between a control LV incorporating a Vesicular Stomatitis virus glycoprotein (VSV-G) and LV pseudotyped with CoV-2 S^{ΔERR}, with or without the prevalent D614G mutation. The D614G mutation was found to increase the titer by approximately 60% (**Figure 2C**). Titration calculations are presented in Table 1 as an example.

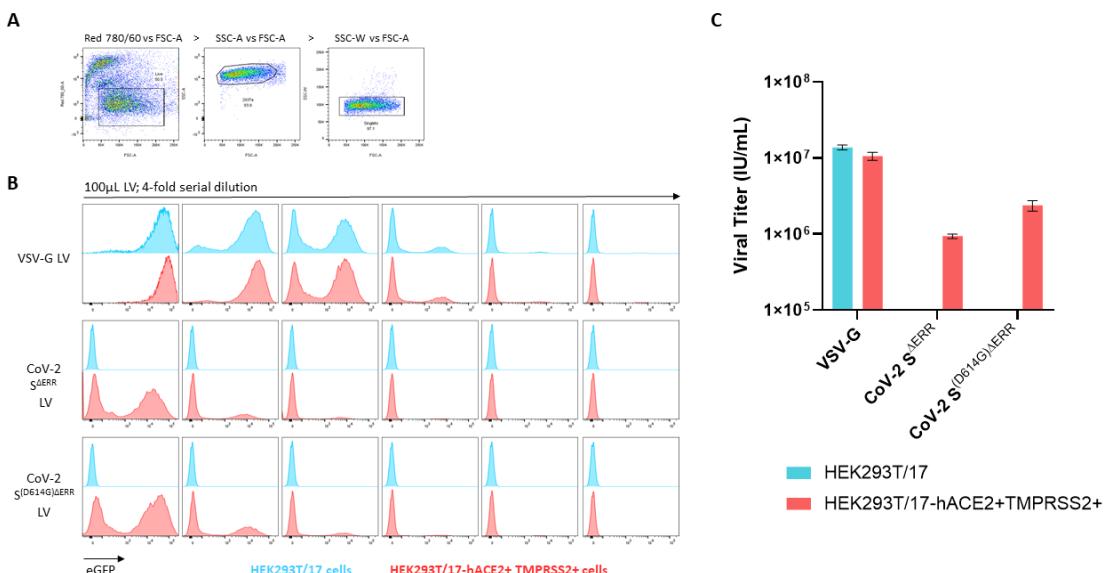


Figure 2. Flow cytometric analysis of pseudotyped lentiviral vectors on stable HEK293T/17-hACE2+TMPRSS2+.

(A) Gating strategy starting with live (eF780) vs. FSC-A to identify live cells, followed by SSC-A vs. FSC-A to identify HEK293T/17 cells, and by SSC-W vs. FSC-A for singlets identification. (B) Transiently produced VSV-G LV, S^{ΔERR} CoV-2 LV, and S^{(D614G)ΔERR} CoV-2 LV were serially diluted from 100 μ L in a 4-fold dilution for six points and added on control HEKT293T/17 cells (blue population) and CoV-2 S permissive HEK293T/17-hACE2+TMPRSS2+ cells (red population) in the presence of 8 μ g/ml of polybrene. Staggered histograms of transduced populations are presented. (C) Graph representing viral titers of the three pseudotyping glycoproteins quantified by an infectivity assay on HEK293T/17 and HEK293T/17-hACE2+TMPRSS2 cells. Data presented are \pm SD of duplicate determinations.

3. Calculate the functional titer as infectious units per ml (IU/ml) using the following equation:

$$\text{Viral titer } \left(\frac{\text{IU}}{\text{ml}} \right) = \frac{(\text{no. of cells seeded at Day 0} \times \left(\frac{\% \text{ of transduced cells}}{100} \right))}{\text{Vector volume (ml)}}$$

Note: For a more accurate titer, take the average of multiple dilutions.

Table 2. Calculation of lentiviral vector titers.

Viral titer calculation of lentiviral vector pseudotyped with VSV-G, CoV-2 S^{AERR} LV, and CoV-2 S^{(D614G)AERR} infected on both HEK293T/17 cells and HEK293T/17-hACE2+TMPRSS2+ cells. Transduction efficiencies higher than 20% and lower than 0.5% are highlighted in grey font and omitted from titer calculation. Average titers are presented from duplicate determinations.

Vector (μl)	HEK293T/17-hACE2+ TMPRSS2+						HEK293T/17					
	VSV-G		CoV-2 S ^{AERR}		CoV-2 S ^{(D614G)AERR}		VSV-G		CoV-2 S ^{AERR}		CoV-2 S ^{(D614G)AERR}	
	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2	n=1	n=2
100	98.7	98.5	50.3	53.2	68.3	66.1	95.7	95.3	0.08	0.031	0.16	0.19
25	93.8	94.9	22	27.1	44.7	40.4	86.8	86.8	0.035	0.025	0.051	0.06
6.25	0	66.2	8.34	11	22.8	18.4	60.5	61.5	0.0088	0.00862	0.00861	0.00871
1.5625	28.2	27.5	2.44	3.36	8.71	6.24	29.4	28.3	0	0	0	0.035
0.390625	8.38	8.47	0.96	0.9	2.58	2.23	11	9.26	0	0	0	0
0.09765625	1.69	2.36	0.29	0.27	0.86	0.74	2.9	2.79	0.00845	0	0	0
IU/ml												
	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-
			6.67	8.80	1.82	1.47						
			E+05	E+05	-	E+06						
					7.81	1.08	2.79	2.00				
					E+05	E+06	E+06	E+06				
	1.07	1.08	1.23	1.15	3.30	2.85	1.41	1.19				
E+07	E+07	E+06	E+06	E+06	E+06	E+06	E+07	E+07				
	8.65	1.21					1.48	1.43				
E+06	E+07	-	-	-	-	-	E+07	E+07				
Average Titer (IU/ml) n=2												
	9.69	1.15	8.92	1.04	3.05	2.11	1.45	1.31				
	E+06	E+07	E+05	E+06	E+06	E+06	E+07	E+07				

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Competing interests

The authors declare no competing interests.

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Thermal Proteome Profiling to Identify Protein-ligand Interactions in the Apicomplexan Parasite *Toxoplasma gondii*

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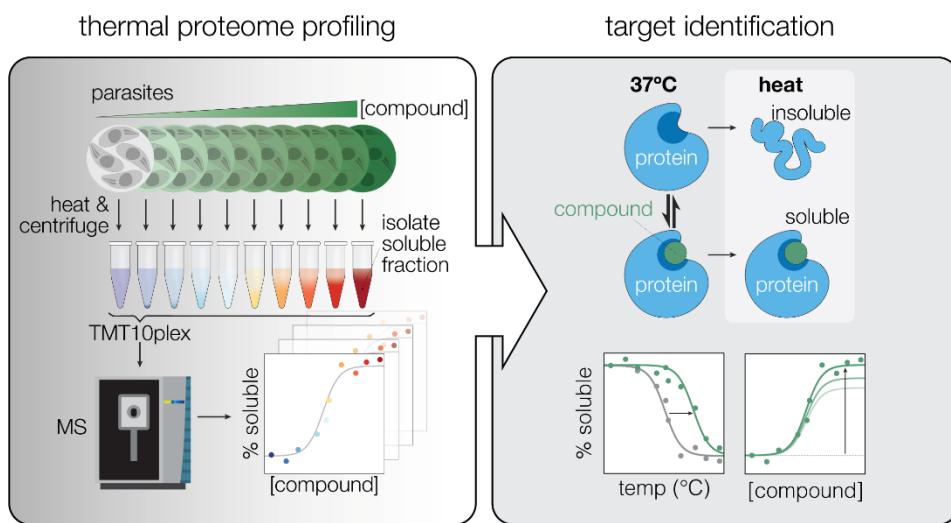
Abstract

Toxoplasma gondii is a single-celled eukaryotic parasite that chronically infects a quarter of the global population. In recent years, phenotypic screens have identified compounds that block parasite replication. Unraveling the pathways and molecular mechanisms perturbed by such compounds requires target deconvolution. In parasites, such deconvolution has been achieved via chemogenomic approaches—for example, directed evolution followed by whole-genome sequencing or genome-wide knockout screens. As a proteomic alternative that directly probes the physical interaction between compound and protein, thermal proteome profiling (TPP), also known as the cellular thermal shift assay (CETSA), recently emerged as a method to identify small molecule–target interactions in living cells and cell extracts in a variety of organisms, including unicellular eukaryotic pathogens. Ligand binding induces a thermal stability shift—stabilizing or destabilizing proteins that change conformationally in response to the ligand—that can be measured by mass spectrometry (MS). Cells are incubated with different concentrations of ligand and heated, causing thermal denaturation of proteins. The soluble protein is extracted and quantified with multiplexed, quantitative MS, resulting in thousands of thermal denaturation profiles. Proteins engaging the ligand can be identified by their compound-dependent thermal shift. The protocol provided here can be used to identify ligand-target interactions and assess the impact of environmental or genetic perturbations on the thermal stability of the proteome in *T. gondii* and other eukaryotic pathogens.

Keywords: Thermal proteome profiling, CETSA, Toxoplasma, Parasite, Proteomics

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Graphical Abstract:



Thermal proteome profiling for target identification in the apicomplexan parasite *T. gondii*.

Background

Target deconvolution is a major challenge for the wealth of compounds identified through phenotypic screening. Chemogenomic approaches, such as directed evolution or drug screens, have been the favored tools for target identification in eukaryotic parasites (Paquet *et al.*, 2017; Cowell *et al.*, 2018; Luth *et al.*, 2018; Rosenberg *et al.*, 2019; Harding *et al.*, 2020). Such approaches require culturing parasites and host cells under compound treatment for extended periods and often identify pathways indirectly affected by a small molecule rather than the target itself. In contrast, several proteomic methods developed in the past decade directly identify interactions between compounds and protein targets (McClure and Williams, 2018; Conway *et al.*, 2021). For example, enrichment of interacting proteins can be performed with derivatized compounds for affinity-purification followed by mass spectrometry (MS). However, these approaches require specialized chemistry and introduce a linker and other chemical groups to the compound of interest, which may affect its behavior.

Thermal proteome profiling (TPP), also known as the cellular thermal shift assay (CETSA), offers a label-free approach that can be performed in a variety of formats that preserve cellular physiology, including *in situ* (Dai *et al.*, 2019; Mateus *et al.*, 2020b). Interactions with a target are identified by a compound-dependent shift in the protein's thermal profile. Cells or cell extracts are treated with the compound and heated to induce thermal denaturation. Aggregated proteins are removed, and soluble proteins are quantified by MS to generate melting curves for each protein. TPP has recently identified the targets of antiparasitic compounds in the apicomplexan parasites *Plasmodium falciparum* (Dziekan *et al.*, 2019; Lu *et al.*, 2020) and *Toxoplasma gondii* (Herneisen *et al.*, 2020), as well as in the trypanosome *Leishmania donovani* (Corpas-Lopez *et al.*, 2019).

The application of TPP extends beyond target deconvolution (Becher *et al.*, 2018; Dai *et al.*, 2018 and 2019; Sridharan *et al.*, 2019; Mateus *et al.*, 2020b). Alterations to protein state and stability may arise from conformational changes, post-translational modifications, altered localization, and interactions with other proteins and biomolecules such as metabolites and nucleic acids. For example, we performed TPP on parasites lacking mitochondrial DegP2 to identify proteins with altered stability based on the loss of this protease (Harding *et al.*, 2020). Genetic perturbations in conjunction with functional proteome profiling are in the early stages (Mateus *et al.*, 2020a) and may be especially well-suited to map the unannotated parts of parasite proteomes.

While TPP has been performed predominantly in mammalian systems, it is expanding to other organisms (Corpas-Lopez *et al.*, 2019; Dziekan *et al.*, 2019; Volkenning *et al.*, 2019; Lu *et al.*, 2020; Herneisen *et al.*, 2020; Harding

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et al., 2020; Jarzab *et al.*, 2020). We believe this approach pairs particularly well with the study of eukaryotic parasites, whose evolutionary divergence complicates identifying molecular pathways by genomic annotation or bioinformatic analysis. For that reason, we provide a detailed protocol describing our thermal profiling pipeline developed for the organism *T. gondii*. Below, we identify key considerations for selecting a TPP workflow appropriate for the researcher's biological question. Step-by-step guidelines follow.

Types of experiment

In this protocol, we stratify steps by choice of material and treatment. TPP can be performed on live parasites or parasite lysates and by melting samples over a range of 10 temperatures ("temperature range") or over a range of 10 compound concentrations melted at a single temperature ("concentration range"). These variations give rise to four permutations described in the *Procedure* as (B) *Lysate Temperature Range Experiment*, (C) *Parasite Temperature Range Experiment*, (D) *Lysate Concentration Range Experiment*, and (E) *Parasite Concentration Range Experiment*. Each experiment has advantages and disadvantages (Franken *et al.*, 2015; Dai *et al.*, 2019; Mateus *et al.*, 2020b). For example, experiments using live cells are more physiological but combine direct and indirect effects. Lysate experiments may more directly identify ligand-protein interactions, but loss of cellular compartmentalization can also lead to non-physiological interactions. Concentration range experiments yield more information-rich thermal profiles, but real interactions may be missed if the thermal challenge temperatures are suboptimal (too low or too high) and the overall coverage of the proteome is reduced due to global denaturation.

Treatment conditions

We have performed thermal profiling experiments on extracellular parasites to avoid the added complexity of the host proteome and confounding effects from compound permeability and host metabolism. Compound treatments are often performed on intracellular parasites; however, the appropriate concentration of compound for the thermal profiling experiment should be determined by assays using extracellular parasites. The thermal profiling experiment should mimic assay conditions as closely as possible. Considerations include the amount of time needed for the compound to arrive at diffusion and binding equilibria, the buffer in which the equilibration takes place, and equilibration temperature (e.g., room temperature vs. 37°C). Mammalian studies have often performed the incubation in PBS (Reinhard *et al.*, 2015; Savitski *et al.*, 2014; Franken *et al.*, 2015). We have used a buffer composition resembling the ionic makeup of the host cytosol (Herneisen *et al.*, 2020; Harding *et al.*, 2020). Buffers should lack serum, which would overwhelm parasite signals that can be quantified by MS.

We aim to process 25 µg of protein per reference sample, which in our experience corresponds to the material from 1×10^7 extracellular parasites of the type I RH strain. We subject concentration-range samples to at least two different thermal challenge temperatures; we have found 54°C and 58°C to work well while still providing sufficient coverage of the proteome. The thermal challenge temperatures may need to be optimized for each experiment; further commentary is provided in the *Data analysis* section.

Lysis conditions

The final lysis buffer composition should contain 0.5-1% IGEPAL CA-630 (also known as NP-40), which provides a balance between solubilizing membrane proteins without re-solubilizing aggregated proteins (Reinhard *et al.*, 2015). For most experiments, the lysis buffer should contain protease inhibitors (and optionally phosphatase inhibitors, depending on the focus of the experiment) and benzonase for digestion of nucleic acids prior to the SP3 cleanup. If the compound of interest is thought to affect proteases, phosphatases, or nucleic acid binding activity, then these supplements should be omitted until after the *Separation of Soluble and Aggregated Protein*. Our lysis buffers have had an ionic composition similar to PBS (Herneisen *et al.*, 2020) and an intracellular-like buffer (Harding *et al.*, 2020), depending on the application. The ionic composition of the buffer (e.g., presence of ATP and metabolites) can substantially influence the melting behavior of proteins (Lim *et al.*, 2018; Sridharan *et al.*, 2019). The concentration of parasite lysate also influences melting behavior; therefore, it is crucial to count the number of parasites prior to lysis and use a consistent lysis buffer volume for the number of parasites. Following harvest,

parasites should be resuspended at least once in a wash buffer that is similar in composition to the lysis buffer (but lacking detergents) to dilute cell culture contaminants, such as serum proteins.

Materials and Reagents

1. T12.5 flask (*e.g.*, Corning Falcon Tissue Culture Flasks, catalog number: 29185-298)
2. T175 flask (*e.g.*, CELLSTAR® Filter Cap Cell Culture Flasks, catalog number: 82050-872)
3. 15-cm dish (*e.g.*, Corning Falcon® Tissue Culture Dishes, catalog number: 25383-103)
4. Corning® 150 mL Bottle Top Vacuum Filter, 0.22 µm Pore 13.6 cm² CA Membrane (Corning, catalog number: 430624)
5. 50 mL conical tube (Corning, catalog number: 430829)
6. Human foreskin fibroblast (HFF) cells (ATCC, catalog number: SCRC-1041)
7. *T. gondii* cell lines (RH, *e.g.*, ATCC 50838 or PRA-319)
8. *T. gondii* filter (Whatman Pop-Top and Swin-Lok Plastic Filter Holders for 47 mm membrane filter size, *e.g.*, VWR catalog number: 28163-089, with GE Healthcare Whatman Nuclepore Hydrophilic Membrane 3 or 5 µm circles, catalog number: 111112 or 111113)
9. Cell scraper (Corning® Small Cell Scraper, catalog number: 3010)
10. Protein low-bind tube (*e.g.*, Eppendorf™ LoBind Microcentrifuge Tubes, 1.5 mL Thermo Fisher Scientific, catalog number: 13698794)
11. 8-strip PCR tubes (*e.g.*, Genesee Scientific, catalog number: 27.125 U)
12. Thickwall polycarbonate open-top ultracentrifuge tubes (0.2 mL, 7 × 20 mm; Beckman Coulter, catalog number: 343775)
13. Protein low-bind 96-well plate (Eppendorf, catalog number: 951032905)
14. Syringes 20 mL (BD Biosciences, catalog number: 302830)
15. Hydrophobic Sera-Mag Speed Beads (GE Healthcare, catalog number: 65152105050250, ~50 mg/mL, keep at 4°C until use)
16. Hydrophilic Sera-Mag Speed Beads (GE Healthcare, catalog number: 45152105050250, ~50 mg/mL, keep at 4°C until use)
17. DMEM (Thermo Fisher Scientific, catalog number: 11965118, keep at 4°C until use)
18. Newborn Calf Serum USA origin, heat Inactivated, sterile-filtered, suitable for cell culture (Sigma-Aldrich, catalog number: N4762-500ML, keep at -80°C until use)
19. 10 mg/mL gentamicin (Life Technologies, catalog number: 15710072, room temperature)
20. 200 mM L-glutamine (Life Technologies, catalog number: 25030081, keep at -20°C until use)
21. 250 U/µL benzonase (Sigma-Aldrich, catalog number: E1014-25KU, store at -20°C)
22. 100× Halt Protease Inhibitor Cocktail (Life Technologies, catalog number: 87786)
23. IGEPAL® CA-630 viscous liquid (Sigma-Aldrich, catalog number: I3021-50ML)
24. 10× PBS suitable for tissue culture (*e.g.*, VWR, catalog number: 45001-130)
25. DC Protein Assay (Bio-Rad, catalog number: 5000116)
26. Tris(2-carboxyethyl)phosphine (TCEP; Pierce, catalog number: 20490; keep at -20°C until use)
27. Methyl methanethiosulfonate (MMTS; Thermo Fisher Scientific, catalog number: 23011, keep at 4°C)
28. Ethyl alcohol, Pure 200 proof, HPLC/spectrophotometric grade (Sigma-Aldrich, catalog number: 459828-1L)
29. Sequencing-grade trypsin (*e.g.*, Promega, catalog number: V5113, keep at -80°C until use)
30. Triethylammonium bicarbonate buffer 1.0 M, pH 8.5 (Sigma-Aldrich, catalog number: T7408-100ML, keep at 4°C)
31. Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, catalog number: 23290, keep at 4°C until use)
32. TMT10plex Isobaric Label Reagent Set (Thermo Fisher Scientific, catalog number: 90110, keep at -20°C until use)
33. 50% hydroxylamine (Thermo Fisher Scientific, catalog number: 90115)
34. Pierce high pH fractionation kit (Thermo Fisher Scientific, catalog number: 84868, keep at 4°C until use)

35. Ultra-high-performance liquid chromatography (UPLC)-MS acetonitrile (Thermo Fisher Scientific, catalog number: A9561)
36. UHPLC-MS water (Thermo Fisher Scientific, catalog number: W81)
37. Pierce Formic Acid, LC-MS Grade (Thermo Fisher Scientific, catalog number: 28905)
38. DMEM + 3% CFS (see Recipes)
39. PBS (see Recipes)
40. 10% IGEPAL CA-630 (also known as NP-40) (see Recipes)
41. 10× CETSA buffer (see Recipes)
42. CETSA wash buffer (see Recipes)
43. CETSA lysis buffer (see Recipes)
44. 1 M TCEP stock solution (see Recipes)
45. 200 mM MMTS stock solution (see Recipes)
46. Buffer A (see Recipes)
47. Buffer B (see Recipes)

Equipment

1. CO₂ incubator (Thermo Fisher Scientific Forma Steri-Cycle 370, catalog number: 370)
2. Clinical benchtop centrifuge (Eppendorf, model: Centrifuge 5810R, catalog number: 022625101)
3. Microcentrifuge (Eppendorf, model: Centrifuge 5424R [discontinued], alternatives include Centrifuge 5425/5425 R)
4. Minicentrifuge (VWR Galaxy Mini Centrifuge, catalog number: 37000-700)
5. Hemocytometer (VWR Counting Chamber, catalog number: 1517O-173)
6. Thermal cyclers (Bio-Rad C1000 Touch™ Thermal Cycler with Dual 48/48 Fast Reaction Module, catalog number: 1851148 and Bio-Rad C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module, catalog number: 1851197)
7. Benchtop ultracentrifuge (Beckman Ultra MAX [discontinued], alternatives include the Optima MAX-XP and Optima MAX-TL)
8. Thermo mixer (Eppendorf, model: ThermoMixer C, catalog number: 5382000023 with 1.5 ml SmartBlock, catalog number: 5360000038)
9. Magnetic stand (Invitrogen Dynamag 2, catalog number: 12321D)
10. Vacuum centrifuge (Savant™ Universal SpeedVac™ Vacuum System, catalog number: SPD111V and, catalog number: UV5450)
11. Lyophilizer (Labconco FreeZone Triad Freeze Dryer, catalog number: 794001030)
12. Orbitrap mass spectrometer (Thermo Fisher Scientific Q Exactive HF-X [discontinued] or Exploris 480, catalog number: BRE725533) with optional FAIMS Pro Interface (Thermo Fisher Scientific, catalog number: FMS02-10001)
13. MS-coupled LC system (Thermo Fisher Scientific EASY-nLC 1200, catalog number: LC140) with Acclaim PepMap 100 75 µm × 2 µm nanoViper trapping column (Thermo Fisher Scientific, catalog number: 164946) and PepMap RSLC C18 3 µm, 100A, 75 µm × 15 cm analytical column (Thermo Fisher Scientific, catalog number: ES900)
14. Pierce formic acid, LC-MS grade (Life Technologies, catalog number: 28905)
15. UPLC-MS acetonitrile (Thermo Fisher Scientific, catalog number: A9561)
16. UPLC-MS water (Thermo Fisher Scientific, catalog number: W81)

Software

1. Proteome Discoverer, version 2.4 (Thermo Fisher Scientific)
2. R, version 4.0 or later: <https://cran.r-project.org/>

3. Tidyverse package, version 1.3: <https://cran.r-project.org/web/packages/tidyverse/index.html>
4. TPP package, release 3.12: <https://bioconductor.org/packages/TPP/>

Procedure

This protocol assumes readers are familiar with *T. gondii* parasite and host cell propagation. For standard reviews, see Roos *et al.* (1994) and Jacot *et al.* (2020).

A. Parasite harvest

1. Infect T175 flasks or 15-cm dishes with confluent HFFs with 2×10^7 - 5×10^7 RH tachyzoites each, which is equivalent to parasites from one fully lysed T12.5 flask, 40-48 h before the assay. Enough T175's should be infected to harvest 4×10^8 parasites for the assay. The yield may vary depending on host cell age, parasite strain, and treatment; in our experience, 3-4 15-cm dishes are usually sufficient to achieve this number of parasites.
2. When the parasites have fully lysed from the monolayer, scrape the flask and collect the media containing extracellular parasites. A fully lysed monolayer contains an abundance of extracellular parasites and few remaining attached host cells. Remove host cell debris by passing the media through a 3 μm filter into one 50 ml conical vial per flask or dish.
3. Concentrate the parasite solution by centrifuging the conicals at $1,000 \times g$ for 10 min at room temperature in a centrifuge with swinging bucket rotors. Discard the supernatant. Resuspend the parasite pellet in 1 ml of wash buffer (lysis buffer without detergents, inhibitors, or enzymes) and transfer the parasite suspension to a 1.5 mL protein low-bind tube.
4. Create a 1:500 dilution of the parasite suspension and count using a hemocytometer.
5. Centrifuge the parasites at $1,000 \times g$ for 10 min at room temperature. Discard the supernatant.
6. Depending on the desired treatment, proceed to section (B) *Lysate Temperature Range Experiment*, (C) *Parasite Temperature Range Experiment*, (D) *Lysate Concentration Range Experiment*, or (E) *Parasite Concentration Range Experiment*.

B. Lysate temperature range experiment

1. Parasite lysis
 - a. Resuspend the parasite suspension in 100 μl lysis buffer per 2×10^7 parasites (see lysis considerations in the *Background*). Sufficient parasites (4×10^8) should be harvested for at least 1.1 mL of lysate, with a small amount of excess to account for pipetting error in the steps below.
 - b. Allow lysis to proceed on ice for 15 min with occasional mixing by pipetting.
2. Compound treatment
 - a. Prepare a compound dilution in the lysis buffer at 2 \times the desired final concentration and a vehicle solution with an equivalent amount of DMSO (or appropriate vehicle). Aliquot 550 μL of each solution into a 1.5 mL protein low-bind tube.
 - b. Combine 550 μL of parasite lysate with 550 μL of the 2 \times compound or vehicle solution and gently pipette to mix. The compound is now at the desired final concentration.
 - c. Aliquot 100 μL of the parasite suspension with vehicle or compound into ten labeled PCR tubes corresponding to the anticipated melting temperatures (see below).
 - d. Allow the solution to equilibrate at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
3. Thermal challenge
 - a. Briefly collect the liquid in the bottom of the tubes using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. Place the PCR tubes in the appropriate orientation on the thermal cycler, such that the tubes with

lysate match the desired temperature.

Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added; the precise sequence depends on the temperature gradient that can be achieved by the user's thermal cycler. We have used melting temperatures of 37°C, 41°C, 43°C, 47°C, 50°C, 53°C, 56°C, 59°C, 63°C, and 67°C split across two PCR strip tubes in 48-well thermal cyclers.

- c. Allow denaturation to occur for 3 min.
- d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
- e. Briefly collect evaporated liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
- f. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

C. Parasite temperature range experiment

1. Compound treatment
 - a. Prepare a compound solution at 2× the desired final concentration and a vehicle solution with an equivalent amount of DMSO (or appropriate vehicle). Aliquot 550 µL of each solution into a 1.5 mL protein low-bind tube.
 - b. Combine 550 µL of parasite suspension with 550 µL of the 2× compound or vehicle solution and gently but thoroughly pipette to mix. The compound is now at the desired final concentration.
 - c. Aliquot 100 µL of the parasite suspension with vehicle or compound into ten labeled PCR tubes corresponding to the anticipated melting temperatures (see below).
 - d. Allow the compound to equilibrate with the parasites at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
2. Thermal challenge
 - a. Briefly collect evaporated liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. Place the PCR tubes in the appropriate orientation on the thermal cycler, such that the tubes with lysate match the desired temperature.

Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added; the precise sequence depends on the temperature gradient that can be achieved by the thermal cycler. We have used melting temperatures of 37°C, 41°C, 43°C, 47°C, 50°C, 53°C, 56°C, 59°C, 63°C, and 67°C split across two PCR strip tubes and 48-well thermal cyclers.

- c. Allow denaturation to occur for 3 min.
- d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
3. Parasite lysis
 - a. Briefly collect evaporated liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. Add 20 µL of 6× lysis buffer to each tube and gently pipette to mix (see treatment considerations in the *Background*). Allow the parasites to lyse on ice for at least 15 min.
 - c. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

D. Lysate concentration range experiment

1. Parasite lysis
 - a. Resuspend the parasites suspension in 100 µL lysis buffer per 2×10^7 parasites (see lysis considerations in the *Background*). Sufficient parasites (4×10^8) should be harvested for at least 1.1 mL of lysate, with a small amount of excess to account for pipetting error in the steps below.

- b. Allow lysis to proceed on ice for 15 min with occasional mixing by pipetting.
2. Compound treatment
 - a. Prepare a dilution series of ten concentrations of the compound, including vehicle alone, at 2× the desired final concentration in lysis buffer. Aliquot 110 µL of the 2× compound solution into a PCR tube.

Note: We advise ensuring that the same concentration of vehicle is maintained across all samples by preparing the dilution series into a lysis buffer containing a vehicle concentration equal to that of the highest compound concentration.

- b. Aliquot 110 µL of the parasite lysate into the PCR tubes containing 2× compound solution. The compound is now at the final desired concentration, and the volume in each tube is 220 µL.
 - c. Use a multichannel pipette to gently mix the lysate and transfer half the volume (110 µL) to another set of PCR tubes. There are now two sets of 10 tubes with 110 µL per tube.
 - d. Allow the solution to equilibrate at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
3. Thermal challenge
 - a. Briefly collect the liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. To induce thermal denaturation, place one set of tubes on a thermal cycler pre-warmed to 54°C and the other set of tubes in a deep-well thermal cycler pre-warmed to 58°C (see considerations in the *Background*).

Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added.

- c. Allow denaturation to occur for 3 min.
 - d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
 - e. Proceed to the step described below, F. Separation of Soluble and Aggregated Protein.

E. Parasite concentration range experiment

1. Compound treatment
 - a. Prepare a concentration range of ten compound solutions, including vehicle, at 2× the desired final concentration in lysis buffer. Aliquot 110 µL of the 2× compound solution into a PCR tube.

Note: We advise ensuring that the same concentration of vehicle is maintained across all samples by preparing the dilution series into a lysis buffer containing a vehicle concentration equal to that of the highest compound concentration.

- b. Aliquot 110 µL of the parasite suspension into the PCR tubes containing 2× compound solution. The compound is now at the final desired concentration, and the volume in each tube is 220 µL.
 - c. Use a multichannel pipette to gently mix the parasite suspension and transfer half the volume (110 µL) to another set of PCR tubes. There are now two sets of 10 tubes with 110 µL of parasites in compound solution.
 - d. Allow the compound to equilibrate with the parasites at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
2. Thermal challenge
 - a. Briefly collect the liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. To induce thermal denaturation, place one set of tubes on a thermal cycler pre-warmed to 54°C and the other set of tubes in a deep-well thermal cycler pre-warmed to 58°C (see considerations in the *Background*).

(Background).

Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added.

- c. Allow denaturation to occur for 3 min.
- d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
3. Parasite lysis
 - a. Briefly collect evaporated liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
 - b. Add 20 μ L of 6 \times lysis buffer to each tube and gently pipette to mix (see treatment considerations in the *Background*). Allow the parasites to lyse on ice for at least 15 min.
 - c. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

F. Separation of soluble and aggregated proteins

Below, we describe the two separation methods we have used for isolation of soluble proteins. For more information, see Note 1.

1. Ultracentrifugation method
 - a. Transfer the heat-challenged lysates (a volume of approximately 100 μ L) to ultracentrifuge tubes pre-chilled on a bed of ice.

Note: The minimum volume of these tubes is 100 μ L. Using lower volumes risks unbalancing the centrifuge rotor.
 - b. Load the tubes into a pre-chilled TLA-100 rotor in a benchtop ultracentrifuge (e.g., Beckman Ultra MAX) chilled to 4°C. The TLA-100 rotor can fit up to 20 tubes, which is enough for the two treatment conditions of a temperature-range experiment or two challenge temperatures of a concentration-range experiment. The tubes must be appropriately balanced to avoid damage to the rotor and ultracentrifuge.
 - c. Centrifuge the samples at 100,000 \times g for 20 min at 4°C using an ultracentrifuge. To calculate the appropriate rpm, use the rotor radius specifications and an online calculator such as <https://www.beckman.com/centrifuges/rotors/calculator>.
 - d. Gently remove the rotor, taking care not to disturb the tubes, and immediately transfer the tubes to ice. If available, work in a cold room.
 - e. Remove the top ~80% by volume of the supernatant and transfer to a pre-chilled protein low-bind tube. It is critical not to disrupt the pellet, which contains aggregated proteins and the membranous fraction.
 - f. Proceed to the next section, *F. Protein Cleanup and digestion with the SP3 protocol*.
2. Filter plate method
 - a. Pre-wet the filter plate with 100 μ L of 1 \times lysis buffer (with compound/treatment, if applicable). Place the filter plate on top of a 96-well plate. Centrifuge at 500 \times g in a swinging-bucket centrifuge for 5 min, until the solution passes through the filter and into the 96-well plate. Discard the solution.
 - b. Place the filter plate over a clean protein low-bind or polypropylene 96-well plate. Transfer the heat challenged lysates (~100 μ L) to the equilibrated filter plate and centrifuge at 500 \times g for 5 min at 4°C to separate the soluble protein from aggregates. Soluble proteins pass through the filter into the 96-well plate.
 - c. Transfer the soluble fraction from the 96-well plate to protein low-bind tubes. The volume of the soluble protein solution is reduced relative to the input volume and should be measured prior to the next step, *G. Protein Cleanup and Digestion*.

G. Protein cleanup and digestion with the SP3 protocol

1. Quantify protein abundance
 - a. Determine the protein concentration in the reference sample (37°C for temperature-range experiments and the lowest compound concentration for concentration-range experiments) using a protein quantification assay, e.g., the DC Protein Assay (Bio-Rad), according to the manufacturer's instructions. Diluent solutions should contain the compound of interest or vehicle, if applicable, as it may substantially alter absorbance readings. The amount of protein determined in this step will be used to calculate the amount of SP3 beads to use for sample cleanup and trypsin to add for digestion. We typically quantify 20-60 µg of soluble protein in the reference sample. The following steps assume a yield of 50 µg in the reference sample; adjust volumes accordingly for lower amounts of protein.
 - b. Transfer a volume corresponding to 50 µg of protein in the reference sample to a new protein low-bind tube. Transfer the same volume of the remaining samples to protein low-bind tubes as well. Raise the volume to 100 µL with lysis buffer.
2. Reduce cysteines
 - a. Add 0.5 µL of a 1 M TCEP solution to each sample. The concentration of TCEP is now 5 mM.
 - b. Incubate the samples at 55°C for 10 min, e.g., on a heat block or thermomixer.
3. Alkylate cysteines
Remove the tubes from 55°C and allow them to cool to room temperature. Add 7.54 µL of a 200 mM MMTS stock solution to bring the concentration to 15 mM. Allow the reaction to occur for 10 min at room temperature.

Note: alternative protocols alkylate with iodoacetamide (IAA) in the dark. We prefer MMTS for in-solution digests due to its rapid reaction rate, stability, and lower non-specific alkylation (Müller and Winter, 2017), which can increase the number of peptide identifications following MS analysis. The choice of alkylating agent will determine search modification on cysteine, i.e., methylthio (+45.988 Da) for MMTS or carbamidomethyl (+57.021 Da) for IAA.

4. Clean up samples using the SP3 protocol (Hughes *et al.*, 2019). For more information, see Note 2.
 - a. Prepare enough hydrophobic and hydrophilic Sera-Mag beads at 50 µg/µL for a 1:10 bead/protein (wt/wt) ratio relative to the reference sample. For example, to process 10 samples with a 50 µg reference sample, prepare 5 mg of beads.
 - i. In a 1.5 mL tube, combine 50 µL of the 50 mg/ml hydrophobic beads with 50 µL of the 50 mg/mL hydrophilic beads.
 - ii. Place the beads on a magnetic rack and allow them to separate. Use a P200 pipette to remove and discard the supernatant.
 - iii. Wash the beads in 100 µL MS-grade water. Place the beads on a magnetic rack and again discard the supernatant.
 - iv. Resuspend the beads in 100 µL of MS-grade water for a final concentration of 50 µg/µL.
 - b. Add 10 µL of 50 µg/µL beads to each sample. The bead/protein (wt/wt) ratio is now at least 10:1.
 - c. Bind the proteins to the beads by adding a 4× volume of 100% HPLC-grade ethanol. For example, to the combined volume of 100 µL sample with 0.5 µL TCEP, 7.54 µL MMTS, and 10 µL Sera-Mag beads, add 472 µL 100% ethanol.

*Note: The protein solution is now 80% ethanol by volume. We found this proportion to be optimal for binding of *T. gondii* proteins to the Sera-Mag beads.*

- d. Allow the proteins to aggregate with the beads by placing the tubes in a thermomixer and shaking at 1,000 rpm at 24°C for at least 10 min. The beads should “clump” upon binding protein.
- e. Place the tubes on a magnetic rack and allow the beads to separate, which takes approximately 30 s. Discard the supernatant into a waste stream that is appropriate for 80% ethanol.
- f. Wash the beads three times with 180 µL 80% ethanol, which can be prepared by diluting HPLC-grade

ethanol with HPLC-grade water. Each time, allow the beads to magnetically separate from the solution for 30 s and dispose of the supernatant into an appropriate waste stream.

Note: In the final rinse, remove as much of the ethanol wash solution as possible to minimize carryover during the enzymatic digestion step. We remove nearly all of the liquid by centrifuging the beads at 16,000 × g for 30 s and double-stacking a P200 and P10 tip to remove the supernatant.

5. Digest proteins into peptides
 - a. Prepare a trypsin digest solution in 50 mM TEAB at a 1:50 (wt/wt) protein:trypsin ratio. Prepare enough stock solution for the number of samples to be processed, e.g., 20 samples for a temperature range experiment with a control and treatment condition and with 10 melting temperatures each.
 - b. Add 35 µL of digest solution to each tube. Gently move the beads into the liquid with the tip of a pipette, but avoid pipetting the beads, as they are sticky.
 - c. Place the tubes in a thermo mixer warmed to 37°C and shake at 1,000 rpm overnight (16-18 h).
 - d. Centrifuge the tubes at 16,000 × g for 1 min at room temperature to pellet the beads and collect evaporated liquid.
 - e. Place the tubes on a magnetic rack and allow the beads to separate for 30 s. Transfer the aqueous supernatant, which contains the digested peptides, to a new protein low-bind tube.

After the peptides have been eluted, samples can be snap-frozen in liquid nitrogen and dried in a lyophilizer with a condenser temperature of -80°C and chamber pressure of approximately 0 mbar. The peptides are typically lyophilized to a powder in four hours or fewer. The lyophilized peptides can be stored at -80°C for several months.

H. Tandem mass tag labeling

Sample multiplexing is performed with isobaric mass tags, which are commercially available in 10-plex and 16-plex format (Werner *et al.*, 2014; Li *et al.*, 2020). We keep working stocks of TMT 10-plex reagents at concentrations of 6.66 µg/µL (100 µg per 15 µL) in acetonitrile at -80°C for 3-6 months. We perform labeling at 2:1 (wt/wt) TMT:peptide (Zecha *et al.*, 2019).

A TMT labeling scheme should be selected in advance of labeling. Each temperature or concentration is labeled with one TMT channel. Two full 10-plex labeling reactions are performed per experiment: the 10 melting temperatures with vehicle and compound for temperature-range experiments or the 10 compound concentrations melted at two temperatures for concentration-range experiments. We have observed reporter ion interference when labeling sequentially (Brenes *et al.*, 2019). Therefore, the labeling schemes shown in **Figure 1** are recommended.

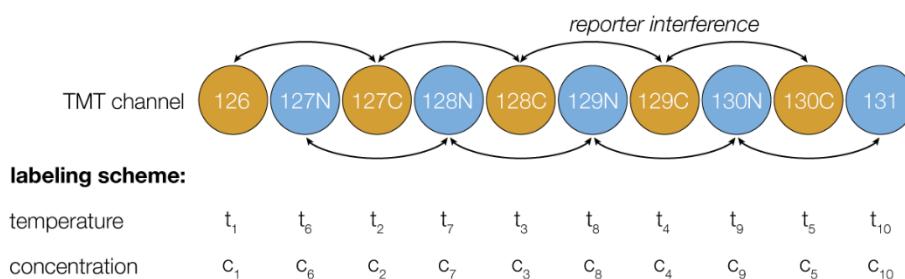


Figure 1. Recommended TMT labeling strategy for temperature- and concentration-range experiments.
t₁/c₁ refers to the lowest temperature or concentration in the experiment.

1. Quantify peptide abundance with the Pierce fluorometric peptide assay

Before starting, quantify the number of peptides in the reference sample (37°C for temperature range experiments and the lowest compound concentration for concentration range experiments) using the Pierce Fluorometric Peptide Assay according to manufacturer's instructions. If peptides have been lyophilized, resuspend in 35 µL 50 mM TEAB pH 8.5. A 1:20 dilution is often sufficient to place the sample within the range of the standard curve, e.g., 0.5 µL of sample + 9.5 µL water. For a standard whole-proteome TMT reaction, use 25-50 µg of peptides in 35 µL of 50 mM TEAB, diluting the sample as necessary. Use equivalent volumes and dilutions of the non-reference samples. The steps below are written for samples containing 50 µg of peptides in 35 µL.

2. TMT labeling reaction

- a. Equilibrate the TMT reagents at room temperature for 3 min.

Note: Record the reagent lot number and isotopic corrections for the batch. This information may be used to create a custom quantification method in Proteome Discoverer that corrects for isotopic impurities arising from natural carbon isotopes.

- b. Centrifuge the TMT reagents at 13,000 × g for 1 min and resuspend each vial in 120 µL of 100% MS-grade acetonitrile. Create 15 µL aliquots and store at -80°C for up to 6 months.

Note: If resuspended TMT reagents will not be used for extended periods of time, lyophilize the reagents and store as a powder at -20°C.

- c. Add 15 µL of TMT reagent (100 µg) to the reference sample (50 µg protein in 35 µL 50 mM TEAB). If working with more or less peptide input, maintain the final vol/vol ratio of acetonitrile (TMT reagents) to aqueous buffer (TEAB buffer). For example, if labeling only 25 µg of peptides in 35 µL of TEAB, add 50 µg of TMT reagent in 7.5 µL and 7.5 µL of 100% acetonitrile to bring the final composition to 30% vol/vol acetonitrile.

- d. Centrifuge the tubes at 13,000 × g for 30 s to collect the liquid.

- e. Place the tubes in the thermomixer and shake at 400 rpm for 60 min at room temperature.

- f. Quench unreacted TMT reagent by adding 3.2 µL of 5% hydroxylamine per 50 µL reaction. Place the tubes in the ThermoMixer and shake at 400 rpm for 15 min at room temperature.

- g. Combine the samples in a 1.5 mL protein low-bind tube. Use the same pipette tip for all transfers to avoid losing peptides due to contact with new surfaces. The volume should now be approximately 530 µL.

- h. Flash-freeze the pooled sample in liquid nitrogen and lyophilize until dry. Note: sample volume may alternatively be reduced via vacuum centrifugation.

- i. Dry samples may be stored at -80°C for several months.

3. Desalting and fractionation

TMT-labeled samples should be fractionated prior to MS data acquisition to reduce isolation interference during MS analysis. We perform high pH reversed-phase peptide fractionation using HPLC (e.g., with Shimadzu LC-20AD; see Herneisen *et al.*, 2020) or the Pierce High pH Reversed-Phase Peptide Fractionation Kit according to manufacturer's instructions, which we have found provide equivalent coverage of the *T. gondii* proteome and also function as a desalting step. We pool samples into eight fractions for LC-MS. The fractions can be lyophilized and stored at -80°C indefinitely.

I. MS data acquisition

Data acquisition methods are highly dependent on facilities. At a minimum, TMT-labeled samples should be acquired using sufficient resolution to resolve the reporter ions and with a long gradient to separate the complex peptide mixtures and reduce co-isolation interference. Here, we describe the data acquisition protocol for our Exploris 480 orbitrap with FAIMS Pro interface coupled to an Easy-nLC 1200 system.

1. Sample resuspension and injection

- a. Resuspend each lyophilized fraction in Buffer A to an estimated concentration of 0.5-1 µg peptides/µL. We typically resuspend each sample in 25 µL. Ensure that the lyophilizate is completely solubilized; it may help to thoroughly wash the sides of the tube and collect the liquid by centrifuging at 16,000 × g for 1 min.
 - b. Transfer each resuspended fraction to an autosampler tube. Once the samples are resuspended, they should be kept at 4°C.
 - c. Inject 0.5-1 µg of peptides for MS analysis (typically 1-2 µL). Samples belonging to the same TMT labeling experiment can be injected sequentially (*i.e.*, the set of fractions). We perform a blank injection between different TMT labeling experiments to reduce carryover.
2. LC gradient
Our samples are separated over a 90-min gradient described in **Table 1**. The gradient includes an optional 12-minute seesaw for column maintenance. Our LC system includes a commercial trapping column (Acclaim PepMap 100 75 µm × 2 µm nanoViper) connected to a 15 cm commercial analytical column (PepMap RSLC C18 3 µm, 100A, 75 µm × 15 cm).
 3. MS acquisition settings
Method parameters for the orbitrap Exploris 480 with FAIMS Pro interface are summarized in Table 2. In our experience, alternating between compensation voltages of -50 and -65 yielded best coverage of the *T. gondii* proteome. The ddMS2 resolution of 30,000 has been optimized for the TurboTMT scan option (Bekker-Jensen *et al.*, 2020); users who elect not to use this setting should opt for a higher resolution.

Table 1. LC gradient used for TMT10-labeled *T. gondii* proteome

Time	Duration	%B
00:00	00:00	1
01:00	01:00	6
42:30	41:30	21
63:15	20:45	36
73:30	10:15	50
74:00	00:30	100
88:00	14:00	100
91:00	03:00	2
94:00	03:00	2
97:00	03:00	98
100:00	03:00	98

Table 2. MS acquisition settings for Orbitrap Exploris 480 with FAIMS Pro interface

Parameter	Setting
Global	
Ion source	
Ion Source Type	NSI
Spray Voltage	Static
Positive Ion (V)	1800
Gas Mode	Static
Ion Transfer Tube Temp (°C)	270
FAIMS Mode	Standard Resolution
FAIMS Gas	Time Dependent
FAIMS Gas Table	
0 min	3 L/min gas
1 min	0 L/min gas
MS Global Settings	
Infusion Mode	Liquid Chromatography
Expected LC Peak Width (s)	30
Advanced Peak Determination	False
Default Charge State	2
Internal Mass Calibration	Off
EXP 1: TMT MS2 FAIMS – 50 CV	
Full Scan	
Orbitrap Resolution	120000
Scan Range (m/z)	350-1200
FAIMS Voltages	On
FAIMS CV (V)	-50
RF Lens (%)	40
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	Disabled
Intensity	

Filter Type	Intensity Threshold
Intensity Threshold	5.0e3
Charge State	
Include charge state(s)	2-5
Include undetermined charge states:	False
Dynamic Exclusion	
Dynamic Exclusion Mode	Custom
Exclude after n times	1
Exclusion duration (s)	30
Mass tolerance	10 ppm
Exclude isotopes	True
Perform dependent scan on single charge state per precursor only	True
Precursor Fit	
Fit threshold (%)	70
Fit window (m/z)	0.7
Data Dependent	
Data Dependent Mode	Cycle Time
Time between Master Scans (sec)	2
ddMS ²	
Multiplex Ions	False
Isolation Window (m/z)	0.7
Isolation Offset	Off
Collision Energy Mode	Fixed
Collision Energy Type	Normalized
HCD Collision Energy (%)	36
Orbitrap Resolution	30000
TurboTMT	TMT Reagents
Scan Range Mode	Define First Mass
First Mass (m/z)	110
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Centroid

EXP 2: TMT MS2 FAIMS – 65 CV

Full Scan		
Orbitrap Resolution	120000	
Scan Range (m/z)	350-1200	
FAIMS Voltages	On	
FAIMS CV (V)	-65	
RF Lens (%)	40	
AGC Target	Standard	
Maximum Injection Time Mode	Auto	
Microscans	1	
Data Type	Profile	
Polarity	Positive	
Source Fragmentation	Disabled	
Intensity		
Filter Type	Intensity Threshold	
Intensity Threshold	5.0e3	
Charge State		
Include charge state(s)	2-5	
Include undetermined charge states:	False	
Dynamic Exclusion		
Dynamic Exclusion Mode	Custom	
Exclude after n times	1	
Exclusion duration (s)	30	
Mass tolerance	10 ppm	
Exclude isotopes	True	
Perform dependent scan on single charge state per precursor only	True	
Precursor Fit		
Fit threshold (%)	70	
Fit window (m/z)	0.7	
Data Dependent		
Data Dependent Mode	Cycle Time	
Time between Master Scans (sec)	2	
ddMS ²		
Multiplex Ions	False	
Isolation Window (m/z)	0.7	

Isolation Offset	Off
Collision Energy Mode	Fixed
Collision Energy Type	Normalized
HCD Collision Energy (%)	36
Orbitrap Resolution	30000
TurboTMT	TMT Reagents
Scan Range Mode	Define First Mass
First Mass (m/z)	110
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Centroid

Data analysis

A. Protein quantification with Proteome Discoverer

Following MS data acquisition, RAW files are processed using any of several analysis pipelines to obtain protein quantification from the MS/MS scans and reporter ion abundances. This protocol describes data processing using the Proteome Discoverer 2.4 software. Alternatives are documented elsewhere (Perez-Riverol *et al.*, 2014; Franken *et al.*, 2015).

1. Load the data into Proteome Discoverer 2.4 by creating a new study and analysis.
 - a. Select processing and consensus workflows that are appropriate for the instrument used for data acquisition and the reporter ion-based quantification method. We use the common templates provided by Thermo Fisher for the Q Exactive for our orbitrap platforms.
 - b. Select TMT 10-plex as a quantification method. We create custom quantification methods with lot-specific corrections, but the default quantification method will suffice.
 - c. Add the RAW spectrum files as fractions (if following the protocol here, add 8 RAW files per experiment). We analyze each set of fractions separately. For example, the vehicle treatment of a temperature range experiment would be analyzed separately from the compound treatment.
2. Adjust settings in the Processing Workflow to conform to the experiment. We use default settings for the Minora Feature Detector, Spectrum Selector, and Percolator nodes (strict targeted FDR of 0.01 based on q-value with a relaxed FDR of 0.05). Major adjustments to the Sequest search engine node include
 - a. Inputting the correct protein database (for *T. gondii* RH strains, the most recent release of the GT1 annotated proteins *.fasta, which can be found at https://toxodb.org/toxo/app/downloads/Current_Release/TgondiiGT1/fasta/data/).
 - b. Selecting the desired dynamic modifications. We have used Oxidation (+15.995 Da) on M, Phosphorylation (+79.966 Da) on S/T/Y, and Acetylation (+42.011 Da) on the N terminus of the protein. Including additional dynamic modifications will increase the search space but may be common practice based on the conditions used in the protein workup steps.
 - c. Selecting the appropriate static modifications: TMT 6-plex (+229.163 Da) on the peptide N terminus and K, and methylthio (+45.988 Da) on C. Note that use of other alkylating agents (*e.g.*, IAA) will require an alternative modification on cysteine. For hyperplexing with SILAC, see Note 3.
3. Adjust the settings in the Consensus Workflow to enable downstream processing of melting curves:

- a. Use only unique peptides for quantification.
 - b. Turn off scaling.
 - c. For temperature range experiments, set Normalization Mode to none; it is important not to normalize abundances by channel, as protein abundance is globally decreasing at higher melting temperatures.
 - d. For concentration range experiments, optionally set Normalization Mode to none. Data can be normalized in the TPP R package (see next section). We have also opted to normalize in Proteome Discoverer and forgo normalization in the TPP package.
 - e. Optionally adjust the co-isolation threshold or Average Reporter S/N threshold. Lowering these thresholds may increase quantification but lower data quality.
4. Upon completion of the analysis, export the protein-level quantification as a *.txt file.

B. Curve Fitting

Curve fitting is performed using the TPP R package, which has been extensively documented (Franken *et al.*, 2015; Childs *et al.*, 2019; Kurzawa *et al.*, 2020). Recently, alternative thermal proteome profiling data analysis packages have been proposed (Dziekan *et al.*, 2020), and users may develop their own custom normalization and curve fitting approaches. The output file from Proteome Discoverer must be modified to match the input format of the TPP package. **Tables S1-S4** represent example output from Proteome Discoverer. **Tables S5-S9** show the streamlined tables used as input to the TPP package, and **Table S10** is representative output.

[Table S1. Temperature range, cells, replicate 1 output from the Proteome Discoverer 2.4 software.](#)

[Table S2. Temperature range, cells, replicate 2 output from the Proteome Discoverer 2.4 software.](#)

[Table S3. Temperature range, lysate, replicate 1 output from the Proteome Discoverer 2.4 software.](#)

[Table S4. Temperature range, lysate, replicate 2 output from the Proteome Discoverer 2.4 software.](#)

[Table S5. Temperature range, cells, replicate 1 trimmed input to the TPP R package.](#)

[Table S6. Temperature range, cells, replicate 2 trimmed input to the TPP R package.](#)

[Table S7. Temperature range, lysate, replicate 1 trimmed input to the TPP R package.](#)

[Table S8. Temperature range, lysate, replicate 2 trimmed input to the TPP R package.](#)

[Table S9. An example configuration table specifying the experiments, conditions, and replicates used for curve fitting in the TPP R package.](#)

[Table S10. Example output from the TPP R package.](#)

C. Anticipated Results

In a typical temperature-range experiment, we detect over 3,000 proteins, of which ~80% have quantification values sufficient for curve fitting. Our other proteomics experiments identify 4,600-4,800 proteins, indicating that the thermal challenge inherent to the thermal profiling approach reduces proteome coverage. We perform experiments in biological duplicate. **Figure 2A** reveals replicate variability in calculated protein melting temperatures. To generate a reference dataset, we performed thermal profiling on live parasites or lysates belonging to the *T. gondii* RH/TIR1 strain and hyperplexed the samples with SILAC (Harding *et al.*, 2020; Herneisen *et al.*, 2020); see Note 4. Aggregates were separated using the filter plate method described in section F of the *Protocol*. As observed for other organisms (Jarzab *et al.*, 2020), proteome-wide thermal stability is greater in lysates than in cells (**Figure 2B**). To include thermostable proteins in our analysis, we calculated the numerical area under the curve (AUC) using the trapezoidal rule (**Figure 2C**). In contrast to the melting temperature, which requires at least 50% thermal denaturation, the AUC metric can be calculated for all proteins with complete thermal profiles. **Figure 2D** shows the relationship between melting temperature and AUC.

Such a reference dataset can be used to select temperature ranges and thermal challenge temperatures for experiments involving compound treatment. **Table 3** summarizes the distribution of melting temperatures from parasites and lysates from two different sets of experiments. The first experiment melted parasites or lysates over a temperature range of 37-67°C and separated soluble proteins from aggregates by

ultracentrifugation (Herneisen *et al.*, 2020). The second experiment, presented here, melted parasites or lysates over a temperature range of 41–73°C and separated aggregates with a filter plate. Researchers may reference the distribution most similar to their intended workflow. To detect compound-dependent thermal stabilization, concentration range experiments should be performed slightly above the melting temperature of the protein target under vehicle-treated conditions (Franken *et al.*, 2015). In cases in which the protein target is not known, we have opted to perform the thermal challenges at two temperatures corresponding to the median and third quartile temperatures of the melting distribution. However, melting temperature often depends on the cellular environment. **Figure 2E** and **F** show melting temperatures and AUC values stratified by subcellular assignment by the MS-based LOPIT approach (Barylyuk *et al.*, 2020). Some subcellular structures, such as the tubulin cytoskeleton and 20S subunit of the proteasome, prove particularly thermostable; detecting compound-dependent thermal shifts in proteins belonging to these substructures would require a high thermal challenge temperature. By contrast, proteins in the nucleus and nucleolus tend to be prone to precipitation, and using the median thermal challenge temperature would result in poor quantification and coverage of proteins in these substructures. Other organelles are particularly sensitive to cellular preparation; for example, components of the 60S ribosome co-melt in cells but exhibit disparate melting profiles in lysates. Therefore, researchers should leverage their observations and predictions about a compound's mechanism of action to select the most appropriate thermal profiling parameters.

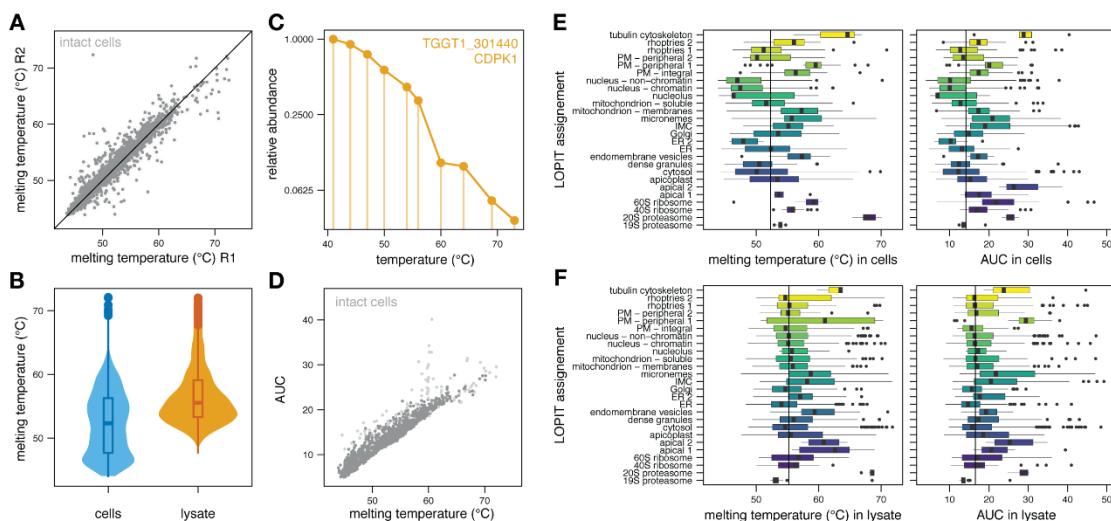


Figure 2. Melting behavior of the *T. gondii* proteome.

(A) Reproducibility of melting temperatures of proteins quantified in both replicates of an intact cell melting experiment. (B) Distribution of average melting temperatures of proteins quantified in both replicates of an intact cell and lysate temperature range experiment. (C) Depiction of how area under the curve (AUC) is calculated by numerical integration using the trapezoidal rule for the protein CDPK1. (D) Relationship between average melting temperature and AUC of proteins for which both values are available. Points with a lighter shade of gray were poorly fit to a sigmoidal melting curve ($R^2 < 0.8$). (E) Distribution of average melting temperatures and AUC in cells or (F) lysates by LOPIT assignment from Barylyuk *et al.* (2020).

Table 3. Distributions of melting temperatures from *T. gondii* lysates and intact cells from two different sets of experiments

Ultracentrifugation (Herneisen <i>et al.</i> , 2020)		Filter plate (here)	
<u>Lysate</u>	<u>Cells</u>	<u>Lysate</u>	<u>Cells</u>
Min	44.8	43.5	47.6
Max	73.0	73.0	73.0
Median	55.0	54.0	55.0
Q1	52.0	51.0	52.0
Q3	58.0	58.0	58.0

1st quartile	50.7	50.9	53.3	47.7
Mean	53.4	53.8	56.7	52.5
Median	52.7	53.4	55.5	52.3
3rd quartile	55.4	56.4	59.1	56.3
Max	65.6	66.9	72.0	72.1

Notes

1. Following thermal challenge and global protein denaturation, soluble protein is separated from unfolded protein aggregates. The original CETSA protocol described centrifugation in a minifuge at $20,000 \times g$ (Jafari *et al.*, 2014), which was subsequently elevated to $100,000 \times g$ in an ultracentrifuge to enhance the signal-to-noise ratio for MS analysis (Franken *et al.*, 2015). Filter plates can be used as an alternative with the benefit of higher throughput (Mateus *et al.*, 2018 and 2020a; Dziekan *et al.*, 2020). After the soluble protein has been separated from the aggregates, samples can be snap-frozen in liquid nitrogen and stored at -80°C for several months.
2. Solutions containing soluble proteins are cleaned up and processed using a modified SP3 protocol based on Hughes *et al.* (2019), which provides high capture and throughput that is well-suited for dilute and low-abundance TPP samples. Protein precipitation is not recommended as it can lead to uneven sample loss that degrades the quality of melting curves. The protocol has been optimized for *T. gondii* protein samples (Harding *et al.*, 2020; Herneisen *et al.*, 2020) and is compatible with TMT-labeling upon elution.
3. To reduce MS time and run-to-run variability, we have hyperplexed TPP experiments using SILAC, as described elsewhere (Herneisen *et al.*, 2020). This variation requires growing parasites in heavy and light SILAC media for 3 passages prior to the TPP experiment. Parasites grown in different media are treated as biological duplicates and are combined in equal weights prior to alkylation. Quantification values originating from the heavy samples are obtained by searching for peptides with heavy arginine (+10.008 Da) and the heavy Lysine-TMT6plex (+237.177 Da) modifications in Proteome Discoverer.
4. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD024912 and 10.6019/PXD024912.

Recipes

1. DMEM + 3% CFS (used for routine parasite passaging) per 500 mL

5 mL 200 mM glutamine
500 μL 10 mg/mL gentamicin
15 mL calf serum

Filter the supplemented DMEM through a bottle top filter into a clean glass bottle that has not been washed with detergent.

2. PBS

100 mL 10 \times tissue culture-grade PBS
900 mL deionized water
Filter-sterilize and store at room temperature

3. 10% IGEPAL CA-630 (also known as NP-40) (50 mL)

5 mL IGEPAL CA-630
45 mL deionized water

Store at 4°C for 6 months

4. 10× CETSA buffer (1 L)

(50 mM NaCl, 1.42 M KCl, 10 mM MgCl₂, 56 mM glucose, 250 mM HEPES pH 7.2)

2.922 g NaCl

105.86 g KCl

2 g MgCl₂

10.1 g glucose

59.575 g HEPES

Add deionized water to 1 L and adjust the pH to 7.2 with KOH

Sterile-filter the solution and store at 4°C

5. CETSA wash buffer (1 mL)

(5 mM NaCl, 142 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 25 mM HEPES pH 7.2)

100 μL 10× CETSA buffer

900 μL deionized water

6. CETSA lysis buffer (1 mL)

100 μL 10× CETSA buffer

80 μL 10% IGEPAL CA-360

10 μL Halt protease inhibitors

1 μL benzonase

809 μL deionized water

7. 1 M TCEP stock solution

1g TCEP HCl

3.489 mL deionized water

Store as 500 μL aliquots at -80°C and as 20 μL working aliquots at -20°C

8. 200 mM MMTS stock solution

200 mM MMTS

7.924 mL isopropanol

Store as 500 μL aliquots at 4°C

9. Buffer A (100 mL)

(0.1% formic acid in MS-grade water)

100 mL MS-grade water

100 μL >99% formic acid

Sonicate for 10 min

10. Buffer B (25 mL)

(80% acetonitrile and 0.1% formic acid)

20 mL MS-grade acetonitrile

25 μL >99% formic acid

5 mL MS-grade water

Sonicate for 10 min

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Competing interests

The authors declare no conflicts or competing interests.

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Extraction and Electrophoretic Analysis of Bacterial Lipopolysaccharides and Outer Membrane Proteins

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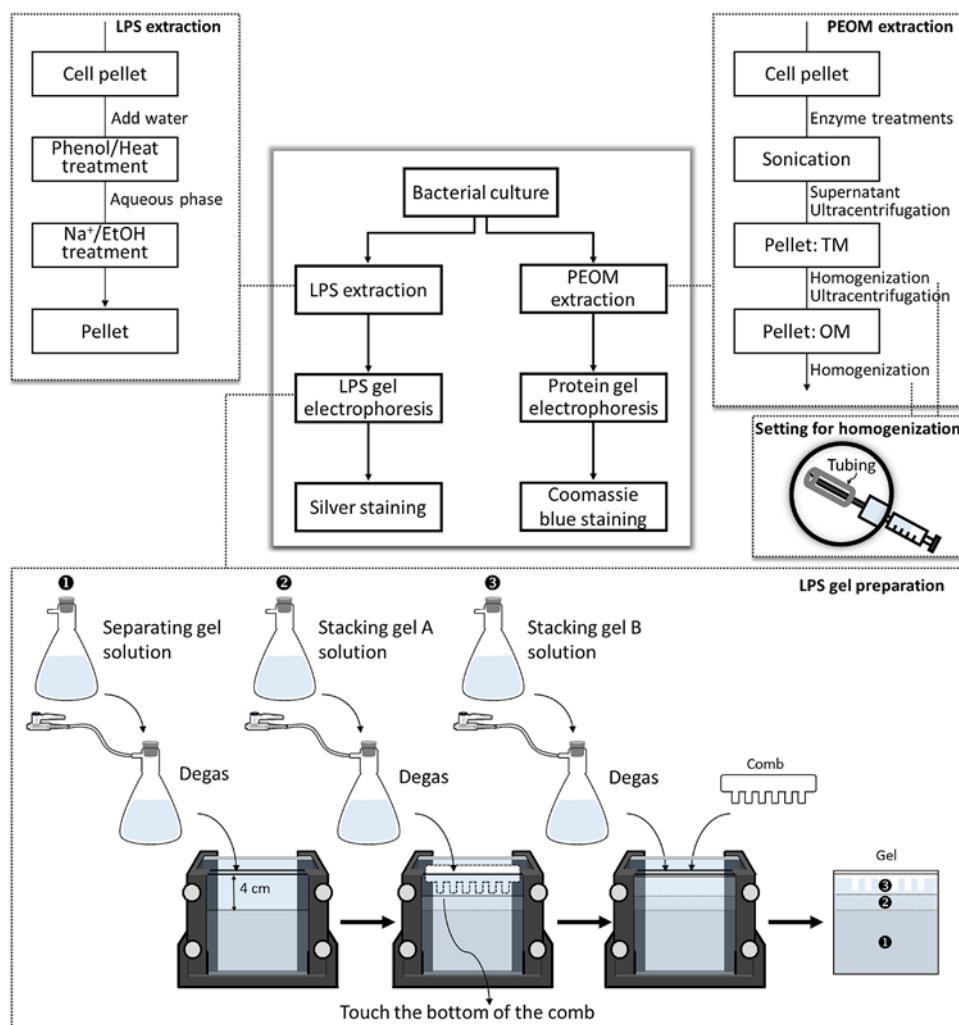
Abstract

Lipopolysaccharides (LPS) (or lipoooligosaccharides [LOS], which lack the O-antigen side chains characteristic of LPS), and outer membrane proteins (OMP) are major cell-surface molecules in the outer membrane (OM) of gram-negative bacteria. The LPS is responsible for causing endotoxic shock in infected hosts and, in conjunction with some OMPs, provides protection to the bacterium against host innate immune defenses and attachment to host cells. Electrophoretic analysis can provide valuable information regarding the size, number, and variability of LPS/LOS and OMP components between bacterial strains and mutants, which aids in understanding the basic biology and virulence factors of a particular species. Furthermore, highly purified extracts are normally not required if only electrophoretic analysis is to be done, and various methods have been established for such procedures. Here, we review ameliorated procedures for fast and convenient extraction of LPS/LOS and protein-enriched outer membranes (PEOM) for optimal electrophoretic resolution. Specifically, we will describe the phenol-based micro-method for LPS/LOS extraction, a differential extraction procedure with sodium lauryl sarcosinate for PEOM, and gel preparation for electrophoretic analysis of LPS/LOS samples in detail.

Keywords: Lipopolysaccharide (LPS), Lipooligosaccharide (LOS), Outer membrane protein (OMP), Protein-enriched outer membranes (PEOM), Phenol-water microextraction, Sodium lauryl sarcosinate, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Silver staining

This protocol was validated in: Infect Immun (2021), DOI: 10.1128/IAI.00567-20

Graphical Abstract:



*Acronyms: LPS = lipopolysaccharide; PEOM = protein-enriched outer membrane; TM = a total membrane preparation including inner membrane, peptidoglycan, and outer membrane; OM = outer membrane.

Workflow for the preparation and analysis of LPS/LOS and PEOM.

Background

The outer membrane (OM) of Gram-negative bacteria is a unique lipid bilayer with two asymmetric leaflets. While the inner leaflet of the OM consists of conventional phospholipids, the outer leaflet predominately contains the lipid A component of lipopolysaccharides (LPS) or lipooligosaccharides (LOS) (Silhavy *et al.*, 2010). LPS molecules typically contain three domains: a hydrophobic membrane anchor known as lipid A (the endotoxic component of LPS), a non-repeating “core” oligosaccharide, and a variable repeating chain of glycoses that make up the O-antigen. LOSs lack the repeating O-antigen of LPS, the core oligosaccharide often branches off different heptose residues, and may phase vary in composition. Furthermore, the terminal oligosaccharide of some species’ LOS can mimic host cell surface oligosaccharides and/or is decorated with sialic acid (Tsai, 2001). LOSs are often present in bacteria that reside predominantly on host mucosal surfaces (Preston *et al.*, 1996). The proteins of the OM (OMP) represent about 50% of the OM mass, and can be divided into integral membrane proteins and

lipoproteins. Unlike the integral IM proteins spanning the membrane in the form of hydrophobic α -helices, integral OM proteins have β sheets that fold into cylinders (Bos *et al.*, 2007). Collectively, LPS/LOS and OMP contribute to the OM's ability to serve as a selective permeability barrier that prevents the entry of harmful substances and allows the influx of nutrient molecules (Nikaido, 2003). Due to their important roles as virulence factors, antigenic factors, and targets for molecular typing, these components are of great interest and have been examined in many biomedical studies (Cloeckaert *et al.*, 1996; Inzana *et al.*, 1997; Giordano *et al.*, 2020) for their roles as adhesins, (endo)toxins, protective barriers to host immunity, and enzymatic proteins.

For detailed studies of the composition of these macromolecules embedded in the OM, it is imperative to prepare highly purified materials and to differentiate subtle changes with sensitive screening methods. LPS/LOS has been successfully extracted with a variety of reagents, including pyridine (Goebel *et al.*, 1945), trichloroacetic acid (Ribi *et al.*, 1961), EDTA (Leive, 1965), phenol (Westphal and Jann, 1965), water (Roberts *et al.*, 1967), ether (Galanos *et al.*, 1969), sodium dodecyl sulfate (SDS) (Darveau and Hancock, 1983), butanol (Morrison and Leive, 1975), chloroform-methanol (Raetz *et al.*, 1985), and methanol (Nurminen and Vaara, 1996). A commonly used method is a variation of the phenol-water extraction procedure (Johnson *et al.*, 1976), which applies EDTA and enzymatic treatment prior to harsh chemical extraction. However, micro versions of the phenol-water protocol (Inzana, 1983) or treatment with proteinase K (Hitchcock and Brown, 1983) are adequate when highly purified materials are not required, such as for electrophoretic gel analysis (Figure 1).

One critical step in the isolation of protein-enriched outer membranes (PEOM) is to separate the OM from the inner (cytoplasmic) membrane (IM) and from proteins in the periplasmic space. Due to differences in the densities of the OM and IM, techniques such as sucrose density gradient centrifugation have been developed for the separation of OM and IM of different bacterial species (Miura and Mizushima, 1968; Osborn *et al.*, 1972; Hancock and Carey, 1979; Loeb *et al.*, 1981). Assays for enzymes specific to the IM (succinic dehydrogenase) can be used to confirm the OM is not contaminated with non-OM proteins (Loeb *et al.*, 1981). However, the IM and OM also differ in their susceptibility to solubilization by detergents. Therefore, a simpler and more rapid approach to isolating PEOM is to use differential extraction with detergents, such as sodium lauryl sarcosinate (Filip *et al.*, 1973; Barenkamp *et al.*, 1981) or Triton X-100 (Schnaitman, 1971; Loeb *et al.*, 1981). Differential gradient centrifugation is ideal for the initial characterization of OM proteins, but extraction with detergent is more time-efficient, sufficient for PEOM preparations, and appropriate for general qualitative and quantitative electrophoretic analysis (Filip *et al.*, 1973; Murakami *et al.*, 2002; Hobb *et al.*, 2009). Different detergents and specific procedures are likely to be more effective for different bacterial species. For example, compared to Triton X-100, we have found sodium lauryl sarcosinate to be more effective for the extraction of *Histophilus somni* PEOM (Figure 2), which can be determined by comparison to PEOM isolated by sucrose density gradients.

The most common and cost-effective analytical method for examination of polysaccharides and proteins is SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Previously, we have reported that optimal gel resolution of LPS/LOS can be achieved by using a bilayer stacking gel and high-quality reagents (Inzana and Apicella, 1999). Other methods have also been described that improve the electrophoretic resolution of LPS/LOS, such as replacing glycine with tricine in the cathode running buffer (Lesse *et al.*, 1990). However, we have not noticed any improvement in resolution using tricine in the cathode buffer compared to the use of two stacking gels. When combined with staining techniques such as silver staining for LPS/LOS (Tsai and Frasch, 1982), and Coomassie blue (Diezel *et al.*, 1972) or silver staining (Merril *et al.*, 1981) for proteins, SDS-PAGE analyses can provide sensitive and relatively rapid information about changes in the structure of LPS/LOS or the expression of OMP. Here, we present protocols for rapid extraction of LPS/LOS and PEOM preparations for electrophoretic analysis. One modification of the protocol previously described for electrophoretic analysis of LPS/LOS (Inzana and Apicella, 1999), is the use of the solubilization buffer described by Tsai and Frasch (1982), which incorporates sucrose in place of glycerol and a lower concentration of 2-mercaptoethanol. These protocols should be applicable to most Gram-negative bacteria.

Materials and Reagents

Notes:

- a. Acrylamide and bisacrylamide are highly neurotoxic. Phenol, sodium lauryl sarcosinate, SDS, ammonium persulfate, TEMED, bromophenol blue, β -mercaptoethanol, and glacial acetic acid are irritants to the eyes, skin, or respiratory tract. Avoid eye/skin contact and inhalation. Handle these compounds while wearing personal protective equipment.
- b. Ethanol, glacial acetic acid, and TEMED are highly flammable. Phenol is combustible. Use these chemicals in a chemical fume hood.

1. Microcentrifuge tubes (1.5-2-mL) (Thermo Fisher Scientific, catalog number: 50-751-5024 or 07-200-210)
2. Conical centrifuge tubes (15- or 50-mL) (Thermo Fisher Scientific, catalog number: 14-959-49B, 06-443-21)
3. Ultra-centrifuge tubes (open-top, thinwall, ultra-clear) and adapters (16 mm diameter) (Thermo Fisher Scientific, catalog numbers: NC9570324, NC1532017)
4. Glass vials (1-fluid dram or smaller size) (Thermo Fisher Scientific, DWK Life Sciences WheatonTM, catalog number: 06-408B)
5. One-milliliter syringe (Thermo Fisher Scientific, Air-TiteTM, catalog number: 14-817-173)
6. Needles (18, 20 and 22 gauge) (Thermo Fisher Scientific, catalog numbers: 14-826-5G, 14-826-5C, 14-826-5A)
7. Plastic tubing (1/32" I.D. \times 3/32" O.D. \times 1/32" Wall; Thermo Fisher Scientific, catalog number: 14-387-347)
8. Acid-washed glass plates (18 cm \times 16 cm; Thermo Fisher Scientific, HoeferTM, catalog number: 03-500-204)
9. Columbia broth (Thermo Fisher Scientific, BD DifcoTM, catalog number: DF0944-17-0), or any medium suitable for growth of the bacteria to be used
10. Columbia blood agar, 5% sheep, 15 \times 100 mm plate (Hardy Diagnostics, catalog number: A16), or any agar plate suitable for growth of the bacteria to be used
11. High-grade distilled water, such as from Milli-Q Integral water purification system
12. HPLC-grade water (VWR, catalog number: 87003-652)
13. Sodium chloride (NaCl) (Thermo Fisher Scientific, J.T. BakerTM, catalog number: 02-004-045)
14. Sodium phosphate monobasic (NaH₂PO₄) (Sigma-Aldrich, catalog number: S3139-250G)
15. Sodium phosphate dibasic heptahydrate (Na₂HPO₄·7H₂O) (Thermo Fisher Scientific, catalog number: S373-500)
16. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: M8266-100G)
17. Trizma[®] base (Sigma-Aldrich, catalog number: T1503-1KG)
18. HEPES (Sigma-Aldrich, catalog number: H3375-250G)
19. Liquid phenol (90%) (Thermo Fisher Scientific, catalog number: A931I-4)
20. Ethanol (Thermo Fisher Scientific, catalog number: BP2818500)
21. Protease inhibitor (Thermo Fisher Scientific, PierceTM, catalog number: PIA32955)
22. DNase I (10,000-25,000 units/mg) (Thermo Fisher Scientific, InvitrogenTM, catalog number: 18-047-019)
23. RNase A (\geq 5000 U/mg protein) (Thermo Fisher Scientific, catalog number: FEREN0531)
24. Sodium lauryl sarcosinate (Sigma-Aldrich, catalog number: L5777-100G)
25. BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, PierceTM, catalog number: PI23227)
26. Acrylamide (VWR, catalog number: VWRV0341-500G)
27. N,N'-Methylene-bisacrylamide (VWR, catalog number: VWRV0172-100G)
28. Urea (VWR chemicals BDH, catalog number: BDH4602-500G)
29. Tetramethylmethylenediamine (TEMED) (Sigma-Aldrich, catalog number: T9281-25ML)
30. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L3771-1KG)
31. Glycine (Sigma-Aldrich, catalog number: G7403-1KG)
32. Ammonium persulfate (Sigma-Aldrich, catalog number: A3678-25G)
33. Sucrose (Sigma-Aldrich, catalog number: S0389)
34. Glycerol (Sigma-Aldrich, catalog number: G5516-500ML)
35. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M3148-100ML)

36. Bromophenol blue (Sigma-Aldrich, catalog number: B8026-5G)
37. 4-12% Bis-Tris gels (Thermo Fisher Scientific, catalog number: NP0336BOX)
38. Protein staining solution (Thermo Fisher Scientific, PageBlue™, catalog number: PI24620)
39. Glacial acetic acid (Thermo Fisher Scientific, catalog number: A38-212)
40. Periodic acid (Sigma-Aldrich, catalog number: P0430-25G)
41. Silver nitrate (Sigma-Aldrich, catalog number: 209139)
42. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Thermo Fisher Scientific, catalog number: C79-500)
43. Ammonium hydroxide solution (NH_4OH) (Sigma-Aldrich, catalog number: 338818-100ML)
44. Sodium Hydroxide (NaOH) (Thermo Fisher Scientific, catalog number: S318-1)
45. Citric Acid monohydrate (Sigma-Aldrich, catalog number: C1909-500G)
46. Formalin (37% formaldehyde) (Sigma-Aldrich, catalog number: 252549)
47. Phosphate buffered saline (PBS, see Recipes)
48. 5 M NaCl (see Recipes)
49. 2 M MgCl_2 (see Recipes)
50. Tris buffers (see Recipes)
51. HEPES buffer (see Recipes)
52. Acrylamide (30%) stock (see Recipes)
53. 9 M Urea solution (see Recipes)
54. SDS solution (10%, see Recipes)
55. 10× Tris/glycine running buffer (see Recipes)
56. 1× SDS Tris/glycine running buffer (see Recipes)
57. 5% Ammonium persulfate solution (see Recipes)
58. 2× Solubilization buffer (see Recipes)
59. Fixative (see Recipes)
60. Oxidizing solution (see Recipes)
61. Silver nitrate solution (20%, see Recipes)
62. Silver stain reagent (see Recipes)
63. Developer solution (see Recipes)

Equipment

1. Milli-Q Integral water purification system (MilliporeSigma, catalog number: ZIQ7010T0)
2. Colorimeter CO7000 (VWR, catalog number: WAPR80-3000-42)
3. Incubator shaker (Thermo Fisher Scientific, Hoefer™, catalog number: SHKE5000 S)
4. Orbital/rocking shaker (Thermo Fisher Scientific, catalog number: 11-676-681)
5. Hot plate with magnetic stirring capability (Thermo Fisher Scientific, catalog number: SP88850200)
6. Sonicator (Qsonica, catalog number: Q500-110)
7. Fume hood (Labconco, catalog number: 100600040)
8. Centrifuges and rotors for handling 0.5-50-mL phenol-resistant centrifuge tubes or bottles [e.g., Beckman Avanti J-E centrifuge (Beckman Coulter, catalog number: A20699) with JA-20 (Beckman Coulter, catalog number: 334831) and JLA-10.500 rotors (Beckman Coulter, catalog number: 369681); Sorvall Legend XTR centrifuge (Thermo Scientific, catalog number: 75210061) with TX-750 rotor (Thermo Scientific, catalog number: 75003180), F13-14x50cy rotor (Thermo Scientific, catalog number: 75003661), round buckets (Thermo Scientific, catalog number: 75003608), and conical adapters (Thermo Scientific, catalog number: 75003639)]
9. Microcentrifuge with a temperature-control system (Eppendorf, catalog number: 5401000137)
10. Ultracentrifuge and rotors (e.g., Optima XE-90 with Type 70.1 Ti rotor, Beckman Coulter, catalog numbers: A94471, 342184)
11. Bottles (250-mL) and adapters for Beckman Avanti J-E centrifuge (Thermo Fisher Scientific, catalog number: NC9304619 and NC1476800)
12. Nephelo culture flasks (Thermo Fisher Scientific, catalog numbers: 50-194-5583 for 300-mL and 50-194-5576

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for 500-mL)

13. Filtering flasks with side arm (vacuum flask) (Thermo Fisher Scientific, KIMAXTM, catalog number: 10-181B)
14. Erlenmeyer Flasks (Thermo Fisher Scientific, PYREXTM, catalog number: 10-090C)
15. Micro stir bars (1/16 × 5/16 inch) (Thermo Fisher Scientific, catalog number: 14-512-150)
16. Spacers (0.75 mm thick, 16 cm long, 2 cm wide; Thermo Fisher Scientific, HoeferTM, catalog number: 03-500-209)
17. Gel casting stand (Thermo Fisher Scientific, HoeferTM, catalog number: 03-500-247)
18. Gel casting frames (Thermo Fisher Scientific, HoeferTM, catalog number: 03-500-241)
19. Gel combs (15 Wells, 0.75 mm thick; Thermo Fisher Scientific, HoeferTM, catalog number: 03-500-188)
20. Polyacrylamide vertical gel electrophoresis system (for LOS/LPS: Hoefer, HoeferTM SE 600, catalog number: SE600-15-1.5) (for PEOM: Thermo Fisher Scientific, InvitrogenTM, catalog number: EI0002)
21. Power supply that is capable of loads from 5 mA and adjustable in 1 mA steps (Bio-Rad, PowerPacTM, catalog number: 1645056)
22. Acid-washed glass dishes or trays (20.1 × 20.1 × 5.5 cm; Thermo Fisher Scientific, catalog number: 15-242B)
23. Gel imaging system (Bio-Rad, ChemiDocTM, catalog number: 17001402)

Procedure

A. Bacterial culture

Note: The culture procedure should be optimized for the bacterium used in the study. The final growth phase for LPS and PEOM extraction should be determined based on the purpose of the study. In general, bacteria grown to at least mid-log phase are preferable for the extraction of LPS and PEOM.

1. Prepare a starter culture at an OD₆₀₀ of 0.8 by inoculating the bacteria freshly isolated from an agar plate into 5 mL of suitable broth in a Nephelo culture flask for LPS extraction, or 25 mL of broth for extraction of PEOM.
2. Dilute the starter culture 1:20 into 5 mL or 200 mL of fresh broth for LPS extraction or PEOM extraction, respectively.
3. Incubate at 37°C at 180 rpm in an incubator shaker for 3 to 4 h, or until the bacteria reach mid-log phase (approximately 10⁹ CFU/mL for *Histophilus somni* and some *Pasteurellaceae* bacteria).
4. Transfer 2 mL of the culture to a microcentrifuge tube and proceed to section B for LPS extraction, or utilize the 200 mL of culture for PEOM extraction (section C).

B. Extraction of LPS/LOS using the hot phenol-water microextraction method (Inzana, 1983)

1. Centrifuge 2 mL of the above bacterial suspension in a microcentrifuge tube at 10,000 × g for 5 min at 4°C.
2. Discard the supernatant and wash the pellet once with 1 mL of PBS.
3. Resuspend the washed cells in 300 µL of distilled water and transfer the suspension to a 1-fluid dram glass vial (or similar) containing a micro stir bar (1/16 × 5/16 inch).
4. Add an equal volume of 90% phenol (pre-warmed to 65-70°C is preferable, but not required) to the bacterial suspension.
5. Place the vials in a beaker of water on a hot plate/stir plate set to 65-70°C. The water should be no higher than half the length of the vials, and multiple vials can be secured together with a rubber band to prevent tipping.
6. Stir the mixture vigorously at 65-70°C for 15 min.
7. Chill the mixture in the vial on ice only enough to cool to about 15°C and transfer the mixture to a 1.5-ml microcentrifuge tube.
8. Centrifuge at 10,000 × g for 10 min at 15°C.

9. Carefully transfer the supernatant to a 15-mL conical centrifuge tube and set the tube aside.
10. Transfer the phenol phase back into the glass vial.
11. Add 300 μ L of water to re-extract the phenol phase and repeat Steps B5 to B9.
12. Pool the aqueous phases in the 15-mL conical centrifuge tube.
13. Adjust the sodium concentration of collected aqueous phases to ~0.5 M by adding one tenth volume of 5 M NaCl.
14. Add 5-10 volumes of cold (-20°C) 95% ethanol to precipitate the LPS and mix the solution thoroughly.
15. Maintain the mixture at -20°C for at least 6 h or overnight.
16. Centrifuge the mixture at 2,000 $\times g$ for 10 min at 4°C.
17. Aspirate the ethanol under vacuum with a pasteur pipette until the tube is dry. Tip the tube to the side with the pellet side up so you do not have to get the pipette tip too close to the pellet.
18. Suspend the opaque pellet in 100 μ L of distilled water.
19. Transfer the 100 μ L of suspension to a 1.5-mL microcentrifuge tube.
20. Repeat Steps B14 to B18 for a second round of precipitation with ethanol.
21. Suspend the precipitate with 50 μ L of distilled water.
22. Store the sample at -20°C. Samples may be stored as aliquots to avoid repeated freeze-thawing.

C. Extraction of protein-enriched outer membranes (PEOM)

1. Centrifuge the 200 mL of bacterial suspension in a 250-mL bottle at 10,000 $\times g$ at 4°C for 15 min.

Note: All steps for extraction of PEOM should be performed at 4°C with protease inhibitor.

2. Wash the pellet twice in 30 mL of 0.05 M Tris buffer, pH 7.8, containing 2 mM MgCl₂ (freeze pellets at -20°C if stopping here).
3. Resuspend the pellet in 25 mL of 10 mM HEPES buffer, pH 7.4, containing protease inhibitor (follow the manufacturer's instruction for the working concentration), and 20 μ g/mL each of DNase I and RNase A.
4. Sonicate the suspension in a 50-mL beaker of ice for 12 bursts of 15 s each, with at least one minute of incubation on ice between each burst (keep below 10°C). Alternatively, two passages through a French Press can be used to lyse the cells (8,000 lbs/in²).
5. Transfer the resultant sample into 50-mL conical tubes.
6. Centrifuge at 12,000 $\times g$ for 5 min to remove unbroken cells.
7. Transfer the supernatant to ultra-centrifuge tubes.
8. Centrifuge the supernatant at 255,000 $\times g$ for 60 min (this yields a total membrane preparation including the IM, peptidoglycan, and OM).
9. Resuspend the pellet in 1 mL of 10 mM HEPES buffer.
10. Homogenize the pellet suspension with a 1-ml syringe using an 18-G, then 20-G, then 22-G needle until thoroughly resuspended. Covering the end of each needle with tubing will prevent scratching the tube (as shown in the graphic abstract). Take care to avoid sudsing.
11. Place aside 0.1 mL of the suspension in a 1.5-mL microcentrifuge tube and freeze at -20°C (do this only once).
12. Transfer the remaining homogenized suspension into ultracentrifuge tubes (~10-mL size).
13. Fill to the top of the tube with 10 mM HEPES buffer containing 2% sodium lauryl sarcosinate. Seal the tubes and tip back-and-forth gently to thoroughly mix.
14. Maintain the tubes at room temperature for 30 min without shaking.
15. Centrifuge at 255,000 $\times g$ for 60 min to obtain PEOM.
16. Remove the supernatant and overlay the pellet with 1 mL of 10 mM HEPES buffer.
17. Repeat Steps C9 to C14, but do not remove 0.1 mL of the suspension again.
18. Resuspend the final pellet in approximately 100-200 μ L of distilled water.
19. Dilute the sample from Step C18 to a protein concentration of 0.5-3 mg/mL, based on the result of BCA protein assay performed following the manufacturer's instructions.
20. Aliquot the sample and store at -20°C for future use.

D. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for LPS/LOS

Note: For optimal resolution of LOS/LPS, use high quality reagents throughout the procedure and prepare fresh if indicated.

1. Preparation of 14% separating gel (20 mL for one 18 cm × 16 cm gel with 0.75 mm spacers)
 - a. Mix 9.3 mL of 30% acrylamide stock, 6.4 mL of 9 M urea, 4 mL of 1.88 M Tris buffer (pH 8.8), and 0.2 mL of 10% SDS in a vacuum flask, gently mix, and degas for 10 min.
 - b. Add 0.15 mL ammonium persulfate solution (5%) and 5 µL of TEMED.
 - c. Gently mix the solution and immediately dispense the separating gel solution between the glass plates to 4 cm below the top of the plate using a syringe or pipette.
 - d. Overlay the gel with 0.1% SDS to a depth of a few millimeters.
 - e. Allow the gel to polymerize for at least 1 h.
 - f. Rinse and blot the top of the gel with filter paper after polymerization.
2. Preparation of 3% stacking gel A (10 mL for one 18 × 16 cm gel with 0.75 mm spacers)
 - a. Mix 1 mL of 30% acrylamide stock, 1 mL of 1.25 M Tris buffer (pH 6.8), 0.1 mL of 10% SDS, and 7.8 mL of HPLC-grade water in a vacuum flask and degas for 10 min.
 - b. Add 0.1 mL ammonium persulfate solution (5%) and 5 µL of TEMED.
 - c. Gently mix the solution and immediately dispense the stacking gel solution between the glass plates to where the bottom of the well comb will be. The bottom of the well comb teeth should just touch the top of the gel after polymerization.
 - d. Allow the stacking gel to polymerize for ~2 h.
3. Preparation of 3% stacking gel B (10 mL for one 18 × 16 cm gel with 0.75 mm spacers)
 - a. Mix 1 mL of 30% acrylamide stock, 1 mL of 10× Tris/glycine running buffer lacking SDS, 0.1 mL 10% SDS, and 7.8 mL of HPLC-grade water.
 - b. Place solution in a vacuum flask and degas for 10 min.
 - c. Insert the comb chosen for the run about half-way between the glass plates.
 - d. After degassing, add 0.1 mL ammonium persulfate solution (5%) and 5 µL of TEMED to the acrylamide solution.
 - e. Gently mix the solution and immediately dispense the stacking gel solution between the glass plates.
 - f. The teeth of the comb should just touch the top of stacking gel A when fully inserted.
 - g. Allow the stacking gel to polymerize for ~2 h.
 - h. The comb can be removed, the wells filled with 1× running buffer, and the gel stored overnight at 4°C after wrapping in plastic wrap.
 - i. Alternatively, only stacking gel A can be used, but resolution of the bands will be reduced.
4. Preparation of LPS/LOS samples
 - a. Add 1-10 µg (depending on the number of bands) of purified LPS/LOS sample or 5 µL of the micro-extract (prepared as above), and dilute to a volume of 10 µL with HPLC-grade water.

Note: If the LPS/LOS gel is overloaded or the development is allowed to proceed for too long, the bands will be obscured, and a smear will form. Normally, 10-12 µg of LPS or 2-3 µg of LOS per well is ideal. If 5 µL of extracted sample is too much or too little, the volume for each sample should be adjusted for a second gel run. Over-development can be prevented or reversed somewhat by addition of 5% acetic acid, but this will also make the bands yellow.

- b. Add an equal volume of 2× solubilization buffer.
 - c. Boil the samples for 5 min by using a floating pad to secure the samples and placing in a beaker of water on a hot plate set to 100°C.
5. Electrophoresis
 - a. Remove the comb and rinse the sample wells with 1× SDS Tris/glycine running buffer, and then fill with the same buffer.
 - b. Add samples carefully, allowing them to sink to the bottom, and not spilling over into other wells.

- c. Place gels into the electrophoresis chamber.
- d. Fill the upper (first) and then lower reservoir with cold (4°C) 1× SDS Tris/glycine running buffer. Two liters is the minimum, 4 L is preferred.
- e. Run the gel at 9 mAmp/gel constant current through the stacking gels, then at 12 mAmp/gel through the separating gel. The total run time will be about 5 h.
- f. When the dye front reaches the bottom of the separating gel, stop the electrophoresis.

E. SDS-PAGE for PEOMs

1. Preparation of protein samples
 - a. Add the PEOM sample to the desired concentration (1-10 µg depending on the number of bands) and dilute to a maximum volume of 7.5 µL (5 µL is preferred) with HPLC-grade water.
 - b. Add an equal volume of 2× solubilization buffer.
 - c. Boil the samples for 10 min by using a floating pad to secure the samples and placing in a beaker of water on a hot plate set to 100°C.
2. Electrophoresis
 - a. Remove the comb from a commercial 4-12% Bis-Tris gel and rinse the sample wells with distilled water.
 - b. Place gels into the electrophoresis chamber.
 - c. Fill the upper buffer chamber and the wells with 1× SDS-Tris/glycine running buffer.
 - d. Add samples allowing them to sink to the bottom.
 - e. Fill the lower buffer chamber with 1× SDS-Tris/glycine running buffer.
 - f. Run the gel at 180 V constant voltage.
 - g. When the dye front reaches the bottom of the gel, stop the electrophoresis.

F. Silver staining for LPS/LOS gels

1. Remove the gel from between the glass plates.
2. Fix the gel in a glass dish with 200 mL of fixative overnight at room temperature covered with plastic wrap; shaking is not required.
3. Discard the fixative.
4. Oxidize the gel in 200 mL of oxidizing solution (made fresh) in the dark by covering the dish with aluminum foil to minimize light exposure.
5. Rotate the dish rapidly (~70 rpm) for 5 min.
6. Discard the oxidizing solution and wash the gel 3 times by rotating slowly with 1 L of distilled water for 15 min each.
7. Prepare the silver stain reagent during the last wash.
 - a. Dissolve 1 g of silver nitrate into 5 mL of distilled water.
 - b. In a separate 500-mL flask, add 2 mL of NH₄OH to 28 mL of 0.1 M NaOH to make the NaOH/NH₄OH solution.
 - c. While gently shaking, slowly add all the silver nitrate solution dropwise to the NaOH/NH₄OH solution. A brown precipitate forms and then becomes clear.
 - d. Add 115 mL of distilled water. (At this point, the reagent should be perfectly clear. If the solution remains brown, do not use it, and make up again with fresh NH₄OH.)
8. Discard the last wash, add the silver stain reagent to the gel, and shake rapidly (~70 rpm) for 10 min.
9. Pour the silver stain reagent into a flask and precipitate with a small amount (large pinch) of CaCl₂ before disposing.
10. Wash the gel 3 times with 1 L of distilled water for 10 min each.
11. Discard the last wash and add 200 mL of the developer solution.
12. Stop the reaction just before optimal development by rinsing the gel in cold water. If over-development occurs, the gel can be destained with 5% acetic acid, but the bands may yellow.

13. Store cold, but photograph or scan as soon as possible.

G. Coomassie blue staining for protein gels

1. Remove the gel from the cassette and place in a clean tray.
2. Wash the gel three times for 5 min each using distilled water with gentle agitation.
3. Discard the last wash.
4. Add a sufficient volume of the Page Blue protein staining solution to cover the gel.
5. Incubate at room temperature for 60 min with gentle agitation.
6. Discard the staining solution and rinse the gel two times with distilled water.
7. Wash the gel three times for 5 min each using distilled water with gentle agitation (or wash more times until the background becomes clear).
8. Store cold until ready to photograph.

Data analysis

A. Profiles of LOSs (lanes 1-4) and LPSs (lanes 5-8) of various genera (Inzana and Apicella, 1999).

1. "Using the reagents described with a single stacking gel, an acceptable profile of LPSs and LOSs was obtained (Figure 1A). However, when a separate comb gel (stacking gel B) containing the same buffer as the upper reservoir buffer was used, the profiles were much sharper, particularly the lower molecular weight LOS bands (Figure 1B)". © 1999 WILEY-VCH Verlag GmbH, Weinheim, Fed. Rep. of Germany.
2. "The number of bands that could be resolved for *Haemophilus* and *Neisseria* species could more accurately be discerned following electrophoresis through the bilayer stacking gel (Figure 1B, lanes 1-4)". © 1999 WILEY-VCH Verlag GmbH, Weinheim, Fed. Rep. of Germany.

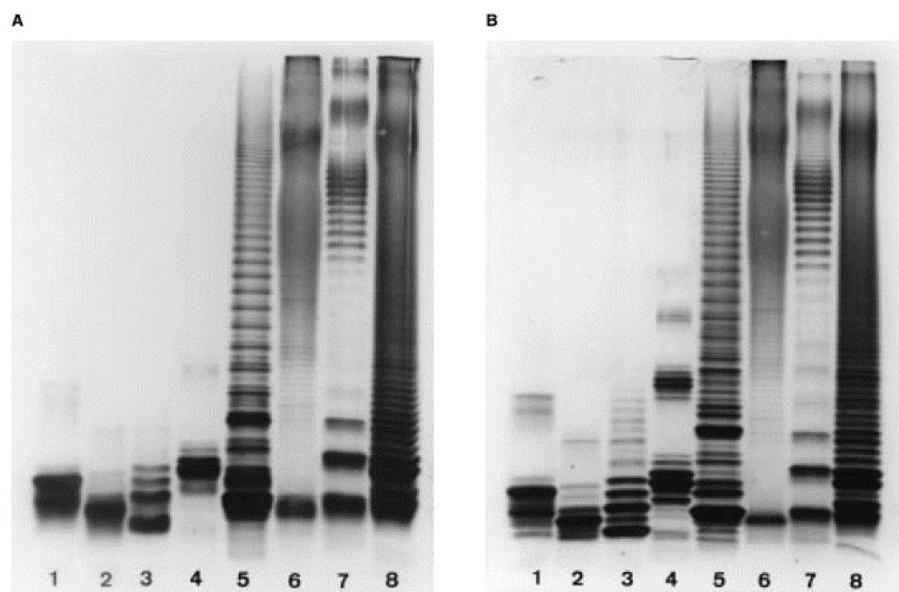


Figure 1. Electrophoretic profiles of LOSs (lanes 1-4) and LPSs (lanes 5-8) of various genera following conventional SDS-PAGE using (A) a single stacking gel or (B) two stacking gels.

Lanes, bacteria from which LOS/LPS samples were isolated, and sample concentration: (1) *Haemophilus*

influenzae type b, 1.5 µg; (2) *Histophilus somni*, 2.2 µg; (3) *Neisseria gonorrhoeae*, 1.0 µg; (4) *Pasteurella multocida*, 1.5 µg; (5) *Actinobacillus pleuropneumoniae* type 7, 7.5 µg; (6) *Brucella abortus*, 12 µg; (7) *Escherichia coli*, 12 µg; (8) *Salmonella typhimurium*, 8 µg. Reproduced with permission from (Inzana and Apicella, 1999) © 1999 WILEY-VCH Verlag GmbH, Weinheim, Fed. Rep. of Germany.

B. Electrophoretic profiles of OMPs from *Histophilus somni* strain (Figure 2).

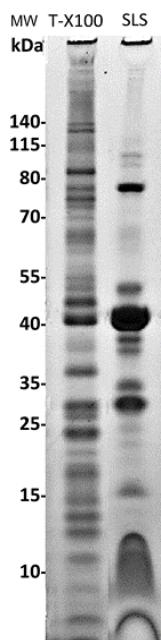


Figure 2. Outer membrane protein (OMP) electrophoretic profiles from *Histophilus somni* strain 2336 following cell lysis and extraction with Triton-X-100 (T-X100) or sodium lauryl sarcosine (SLS). The T-X100 extract contains many proteins associated with the inner membrane, and the SLS extract is enriched for well-known major OMPs, such as the ~40 kDa protein. However, lower molecular size proteins (<15 kDa) were not well resolved using SLS. Molecular size markers (MW) are to the left. Four µg of protein were added/lane indicating there is about 10 ng of protein/band.

Recipes

1. Phosphate buffered saline (PBS)

8.5 g NaCl (150 mM)
0.23 g NaH₂PO₄ (1.9 mM)
2.17 g Na₂HPO₄·7H₂O (8.1 mM)
Add distilled water up to 1 L, adjust pH to 7.4.

2. 5 M NaCl

29.22 g NaCl
Add distilled water up to 100 mL.

3. 2 M MgCl₂

19.04 g MgCl₂
Add distilled water up to 100 mL.

4. Tris buffer (0.05 M, pH 7.8) containing 2 mM MgCl₂

0.61 g Trizma® base

100 µL of 2 M MgCl₂ (add before use)

Add distilled water up to 100 mL, adjust pH to 7.8, store at 4°C.

5. Tris buffer (1.25 M, pH 6.8)

15.14 g Trizma® base

Add distilled water up to 100 mL, adjust pH to 6.8, store at 4°C.

6. Tris buffer (1.88 M, pH 8.8)

22.77 g Trizma® base

Add distilled water up to 100 mL, adjust pH to 8.8, store at 4°C.

7. HEPES buffer (10 mM, pH 7.4)

0.24 g HEPES

Add distilled water up to 100 mL, adjust pH to 7.4.

8. Acrylamide stock (30% T, 0.8% C)

29.2 g acrylamide

0.8 g bis-acrylamide (C, cross-linker)

Add distilled water up to 100 mL. Filter stock solution, wrap in aluminum foil, and store at 4°C.

9. Urea solution (9 M)

54.05 g urea

Add distilled water up to 100 mL and store at room temperature.

10. 10% SDS solution

10 g SDS

Add distilled water up to 100 mL and store at room temperature.

11. 10× Tris/glycine running buffer

30.29 g Trizma® base (0.25 M)

144.13 g glycine (1.92 M)

Add distilled water up to 1 L, store at room temperature.

12. 1× SDS-Tris/glycine running buffer (4 L)

400 mL of 10× Tris/glycine running buffer

40 mL of 10% SDS solution

Add distilled water up to 4 L, store at 4°C.

13. 5% Ammonium persulfate solution

50 mg ammonium persulfate

Add distilled water up to 1 mL (prepare fresh for each experiment).

14. 2× solubilization buffer (100 mL)**For LPS/LOS gels:**

10 mL of 1 M Tris-HCl buffer, pH 6.8 (0.1 M)

2 g SDS (2%)

20 g sucrose (20%)

0.5 mL of 1% bromophenol blue (0.005%)

Add distilled water up to 100 mL.

Just before use, add 10 µL of 2-mercaptoethanol to 990 µL of 2× solubilization buffer.

For OMP gels:

12.5 mL of 1 M Tris-HCl buffer, pH 6.8 (0.125 M)

4 g SDS (4%)

20 mL glycerol (20%)

0.5 mL of 1% bromophenol blue (0.005%)

Add distilled water up to 100 mL.

Just before use, add 100 µL of 2-mercaptoethanol to 0.9 mL of 2× solubilization buffer.

15. Fixative (200 mL)

10 mL glacial acetic acid (final concentration 5%)

80 mL ethanol (final concentration 40%)

110 mL distilled water

16. Oxidizing solution

1.4 g periodic acid (final concentration 0.7%)

200 mL of the fixative

17. 20% Silver nitrate solution

1 g silver nitrate

Add distilled water up to 5 mL.

18. Silver stain reagent (see Procedure section E for details of preparation)

28 mL of 0.1 N NaOH

2 mL of NH₄OH solution

5 mL of 20% silver nitrate (add silver nitrate dropwise)

115 mL of distilled water

19. Developer solution

50 mg of citric acid

0.5 mL of 37% formaldehyde (formalin)

Add distilled water up to 1 L.

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The original work on preparation and analysis of PEOM protocols was derived from Loeb and Smith (1980), Barenkamp *et al.* (1981) and Loeb *et al.* (1981).

Competing interests

The authors have no financial or non-financial competing interests.

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Bacterial Infection and Hypersensitive Response Assays in *Arabidopsis-Pseudomonas syringae* Pathosystem

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Abstract

Arabidopsis thaliana-*Pseudomonas syringae* pathosystem has been used as an important model system for studying plant-microbe interactions, leading to many milestones and breakthroughs in the understanding of plant immune system and pathogenesis mechanisms. Bacterial infection and plant disease assessment are key experiments in the studies of plant-pathogen interactions. The hypersensitive response (HR), which is characterized by rapid cell death and tissue collapse after inoculation with a high dose of bacteria, is a hallmark response of plant effector-triggered immunity (ETI), one layer of plant immunity triggered by recognition of pathogen-derived effector proteins. Here, we present a detailed protocol for bacterial disease and hypersensitive response assays applicable to studies of *Pseudomonas syringae* interaction with various plant species such as *Arabidopsis*, *Nicotiana benthamiana*, and tomato.

Keywords: *Arabidopsis thaliana*, *Pseudomonas syringae*, Disease resistance, Hypersensitive response, Cell death

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Background

Pseudomonas syringae is a Gram-negative phytopathogenic bacterial species that causes diseases on a broad host range, namely bacterial speck in tomato and canker disease in pepper and kiwifruit (Lewis Ivey and Miller, 2000; Basim *et al.*, 2004; Mazzaglia *et al.*, 2012; Xin and He, 2013). Over the last two decades, *Pseudomonas syringae* has also been an important model pathogen for studying bacterial ecology, pathogenesis mechanisms, and plant immune system (Xin *et al.*, 2018). Due to its importance in basic biology research, as well as in outbreaks of economically-important diseases, it was selected as the number one of the top 10 plant pathogenic bacteria in molecular plant pathology (Mansfield *et al.*, 2012).

P. syringae bacteria are generally used as foliar pathogens in laboratories, although in nature they cause diseases in various organs. *P. syringae* enters plant leaf tissue through wounds or open stomata during natural infections, and uptakes nutrients in the apoplastic space of the leaves for multiplication (Xin and He, 2013). Bacterial disease assays are powerful tools in plant pathology studies. Two inoculation approaches, surface inoculation (*i.e.*, by dipping or spray) and infiltration (*i.e.*, by a needle-less syringe or vacuum), are commonly used in laboratories (Katagiri *et al.*, 2002). Here we present step-by-step procedures for bacterial disease assays by syringe infiltration, which bypasses pathogen entry through stomata and plant “stomatal defense”, and is broadly used in studying plant “apoplast defense”. In addition, we also describe detailed procedures of the hypersensitive response assay, in which recognition of pathogen effectors by plant immune receptors triggers fast tissue cell death, and the rate of cell death can be used as a readout of the strength of plant immunity. Although this protocol is presented using the *Arabidopsis-P. syringae* pathosystem, it can be easily adapted to different pathosystems, such as *Nicotiana benthamiana-P. syringae* and tomato-*P. syringae* with slight modifications on bacterial inoculum.

Materials and Reagents

1. Eppendorf tubes (1.5 mL and 2 mL, Thermo Fisher Scientific, catalog number: 509-GRD-Q and 508-GRD-Q)
2. 0.22 µm Millex-GP Syringe Filter (Merck, catalog number: SLGPR33RB)
3. 96-well plate (200 µL, Round Bottom, Beyotime, catalog number: FPT016)
4. Paper towels
5. Pipette tips (Thermo Fisher Scientific QSP, catalog number: 112NXL-Q)
6. 1 mL needleless syringe (LABSTAR, catalog number: BX150)
7. *Arabidopsis thaliana* accession Col-0, *fec* (Gimenez-Ibanez *et al.*, 2009) and *rps2* (Mindrinos *et al.*, 1994)

Note: Arabidopsis Col-0 plant contains the RPS2 gene, which mediates the recognition of effector protein AvrRpt2 and induces plant ETI resistance to Pst DC3000(avrRpt2). fls2 efr cerc1 (fec) triple mutant, which is mutated in three major pattern-recognition receptor genes; rps2 mutant is mutated in the RPS2 gene, encoding the receptor recognizing AvRpt2.

8. *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 and Pst DC3000(*avrRpt2*) (Mudgett *et al.*, 1999)
9. Sodium hypochlorite (Sinopharm Chemical Reagent, catalog number: 80010428)
10. Sterilized water (*e.g.*, Milli-Q)
11. Mixed soil, which contains substrate (PINDSTRUP), vermiculite (Size: 1-3 mm) and perlite (Size: 3-5 mm), the ratio of these materials is 3:9:1 in mixed soil.
12. Tryptone (OXOID, catalog number: LP0042B)
13. Yeast Extract Powder (OXOID, catalog number: LP0021B)
14. Potassium dihydrogen phosphate (KH₂PO₄) (Sinopharm Chemical Reagent, catalog number: 10017608)
15. Sodium chloride (NaCl) (Sinopharm Chemical Reagent, catalog number: 10019318)
16. Magnesium sulfate (MgSO₄) (Sinopharm Chemical Reagent, catalog number: 20025117)
17. Agar powder (Shanghai DingGuo Biotech, catalog number: DH010-1.1)
18. Rifampicin (Yeasen Biotechnology, catalog number: 60234ES08)
19. Spectinomycin (Sangon Biotech, catalog number: A600901-0005)

20. 75% ethanol (Sinopharm Chemical Reagent, catalog number: 80176965)
21. Luria-Marine (LM) solid medium (see Recipes)
22. Rifampicin stock stock (50 g/L, 1,000 \times) (see Recipes)
23. Spectinomycin stock stock (50 g/L, 1,000 \times) (see Recipes)

Equipment

1. Ultra-low temperature freezer (-75°C freezer, New Brunswick Scientific)
2. Tray (size: 310 g), transparent plastic dome, pot (size: 8 cm) and mesh (pore size: mesh 18 = 880 μm)
3. Arabidopsis growth chamber (Percival and JIUPU)
4. Pipette (1 mL, Rainin, model: L-1000PL for export)
5. Centrifuge (Eppendorf, model: 5425R)
6. Spectrophotometer (Thermo Fisher Scientific, model: NanoDrop ONE^C)
7. Steel ball (5 mm in diameter; SSCB, catalog number: KH000268)
8. Millex-GP Syringe Filter Unit (Merck, catalog number: SLGPR33RB)
9. Camera (Canon, model: EOS 80D)
10. Vortex Oscillator (Scientific Industries, model: Vortex-Genie 2)
11. Tweezer
12. Beaker (Thermo Scientific, catalog number: 1201-1234)
13. Cork borer (Sigma-Aldrich, catalog number: Z165220-1SET, 7.5 mm in diameter)
14. TissueLyser (Shanghai Jingxin Industry, model: Tissuelyser-48)
15. Stereoscope (Leica, model: MDG41)
16. Autoclave
17. Temperature and Humidity Data Logger (Easylog, model: EL-21CFR-2-LCD)

Software

1. Microsoft Excel
2. GraphPad Prism 8

Procedure

A. Growing *Arabidopsis* plants in soil

1. Sterilize *Arabidopsis* seeds with 5% sodium hypochlorite for 7-10 min, and then wash the seeds with sterilized water 5 times; place the sterilized seeds in the dark at 4°C for two days.

Note: The cold treatment will synchronize germination.

2. Place the mixed soil in an ultra-low temperature freezer (below -20°C) overnight.
3. Place the soil into small plastic pots, cover the pots with mesh, and fix the mesh with a rubber band (Figure 1A).

Note: Autoclaving soil is usually harmful for seed germination and plant growth in our hands. We therefore used a freezing treatment to kill insect eggs and larvae in the soil to prevent insect infestation during plant growth, without causing any visible effect on seed germination.

4. Use a pipette to sow sterilized seeds (from A1, about 200-300 seeds/mL) in the soil (about 20-30 seeds per pot, 4-8 seeds at each corner).
5. Grow plants in environmentally-controlled growth chambers, with relative humidity set at 60%, temperature at 22°C, light intensity at $100 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a 12 h light/12 h dark photoperiod.
6. Remove excess seedlings after one week, and keep 4 seedlings per pot.
7. Water the plants with tap water every 2-3 days. Four- to five-week-old plants (Figure 1B) were used for our experiments.

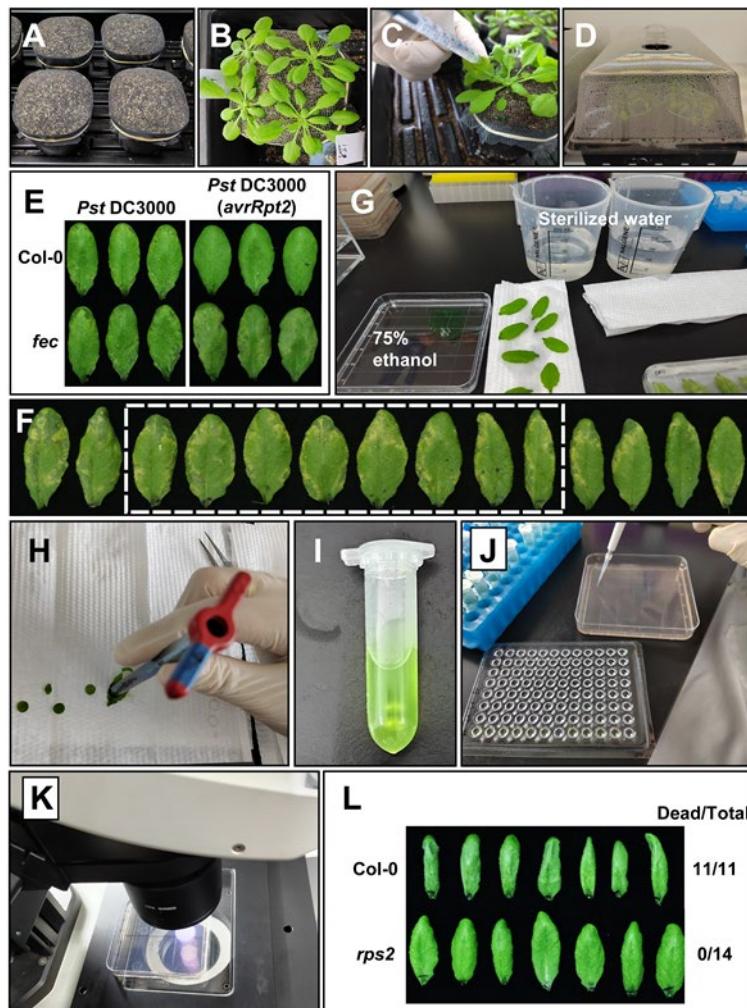


Figure 1. Experimental procedures of bacterial infection and hypersensitive response assays.

A. Preparation of the mixed soil in pots. B. Appearance of 4-week-old *Arabidopsis* plants grown in environmentally-controlled growth chambers. C. Inoculation of leaves with bacterial solutions using a 1 mL needleless syringe. D. Inoculated plants are covered with a dome to keep high humidity, for the disease to develop in the greenhouse. E. Photograph of inoculated leaves 3 days after infiltration. F. Disease phenotype 3 days after infiltration in Col-0 plants, the white dotted box represents selected representative samples. G. Sterilization and rinsing of the sampled leaves. H. Sampling of leaf discs using a cork borer (7.5 mm in diameter). I. Ground leaf solution. J. Dilute the extracted solutions in different dilution ratios, and then take 10 μL from each dilution and place on LM agar plates. K. Count the colonies with a stereoscope. L. Photographs of tissue collapse phenotype for HR assay. *Pst DC3000 (avrRpt2)* bacteria were infiltrated at OD₆₀₀ of 0.2 and images were taken about 7 h post infiltration (hpi).

B. Preparing Pst strains for inoculation

1. Streak out the Pst strains from -75°C freezer onto Luria-Marine (LM) solid medium containing antibiotics, and allow to grow in a 30°C incubator for 2 days.
2. Culture the bacterial strains in 4-6 mL LM liquid medium supplemented with the appropriate antibiotics [50 mg/L rifampicin for *Pst* DC3000; 50 mg/L spectinomycin and 50 mg/L rifampicin for *Pst* DC3000(*avrRpt2*), which contains the *pDSK600-avrRpt2* construct with spectinomycin resistance], shaking at 200 rpm and 30°C for 12-16 h.

Notes: The bacterial culture should reach mid-log growth phase (OD₆₀₀ = 0.6-1.0).

3. Transfer 1.5 mL bacterial culture to a 2 mL Eppendorf tube, and collect by centrifugation at 2,500 × g for 5 min.
4. Remove the supernatant and resuspend the pellet with 2 mL sterilized water to wash.
5. Centrifuge the bacterial solution at 2,500 × g for 5 min, and remove the supernatant, and then resuspend the pellet with 1 mL sterilized water.
6. Adjust the bacterial solution to a cell density of OD₆₀₀ = 0.2 (~1 × 10⁸ cfu/mL) with sterilized water, measured with a spectrophotometer.

For bacterial disease assay

C. Inoculation of *Arabidopsis* with Pst strains

1. Dilute the bacterial solution from B6 with sterilized water to a cell density from OD₆₀₀ = 0.001 (~5 × 10⁵ cfu/mL) to 0.002 (~1 × 10⁶ cfu/mL).
2. Inoculate 3 marked leaves (from the abaxial side of the leaves) per plant with adjusted bacterial solution using a 1 mL needleless syringe (Figure 1C and Video 1). We estimate that approximately 100-200 μL are necessary to fully infiltrate one adult leaf. We usually inoculate 4 plants for each strain, and inoculate different plants with different strains in each pot.
3. Wipe off the solution on the surface of the infiltrated leaves with a paper towel.
4. Keep inoculated plants under ambient humidity for about 1 h to allow evaporation of excess water from the leaf.
5. Cover the tray with a transparent plastic dome to keep high humidity until sampling, and place plants back in the growth chamber for the disease to develop (Figure 1D)



Video 1. Syringe infiltration demonstration.

D. Recording the disease symptoms and counting the number of bacteria

1. Harvest samples after 2-4 days (varies among experiments, due to different plant genotypes/bacterial strains), remove all inoculated leaves from the plants, and take a photo to record the chlorosis and necrosis symptoms (Figure 1E).
2. Select 6-8 leaves that are representative of the symptoms (*e.g.*, the middle level; Figure 1F), and place them in a 75% ethanol solution for ~30 s to kill the bacteria on the leaf surface.
3. Place the leaves on the paper towel to quickly remove excess ethanol, and then rinse the leaves with sterilized water twice (Figure 1G).
4. Dry the leaves with the paper towel, take two leaf discs from each leaf using a cork borer (7.5 mm in diameter) and four discs from two different leaves as one biological repeat, place the leaf discs into a 2 mL Eppendorf tube containing 200 μ L of sterilized water and one or two steel balls (5 mm in diameter); collect three to four repeats from each treatment (Figure 1H).
5. Grind the leaf discs by TissueLyser at 30 Hz, for 1 min.
6. Quickly spin the extracted solutions ($5,000 \times g$, 10 s) to move the solution from the tube caps to the inside of the tube; open the tube and add 800 μ L of sterilized water to the tube, briefly vortex, and mix well by Vortex Oscillator (Figure 1I).
7. Serially dilute the bacterial solutions with sterilized water (*i.e.*, by $10\times$, $100\times$, $1,000\times$, *etc.*), and then take 10 μ L from each dilution and place on LM agar plates supplemented with rifampicin (at 50 mg/L). Perform two technical replicates for each sample (Figure 1J), and air dry the plates at room temperature.

Note: As 10 μ L from 1 mL of the extracted solution were placed on a LM agar plate, this is the equivalent of a 100-fold dilution of the bacteria from 4 leaf discs. If we take 10 μ L from 1 mL of the extracted solution to 90 μ L of sterilized water and then take 10 μ L to place on the LM agar plate, this is equivalent to another 10-fold dilution. Serial 10-fold dilutions are done for each sample by repeating this process. We usually dilute the extracted solution to 10^{-4} , 10^{-5} and 10^{-6} for Pst DC3000, and dilute to 10^{-2} , 10^{-3} and 10^{-4} for Pst DC3000 (avrRpt2).

8. Place the air-dried LM agar plates in an incubator at 30°C for colonies to grow.
9. Count the colonies with a stereoscope 24 h after incubation (Figure 1K). It can also be counted by eye if the colonies are well separated and grow to large sizes (*e.g.*, after more than 24 h incubation).

For the hypersensitive response assay

E. Inoculation of *Arabidopsis* plants with *Pst* strains

1. Inoculate 3-4 marked leaves (from the abaxial side of the leaves) per plant with Pst DC3000(avrRpt2) strain at a cell density of OD₆₀₀ = 0.2 (~ 10^8 cfu/mL) using a 1 mL needleless syringe (Figure 1C). Inoculate 4-6 plants for each genotype.
2. Wipe off the solution on the surface of the leaves with a paper towel, let the leaves dry, and place the plants back in growth conditions without cover and under ambient humidity. Check tissue collapse starting from 4-5 h after infiltration.

Note: Different effector proteins lead to different ETI response intensities and different rates of HR, so different strains may require distinct observation time for HR.

3. Harvest leaves, count the number of leaves showing cell death, and take a photo (Figure 1L).

Note: When cell death occurs, ions will leak out from the cell, and the electrolyte leakage can be measured

using a Electrolytic conductivity meter over a time course after infiltration, providing a more quantitative way of assessing HR (protocols described in Hatsugai and Katagiri, 2018).

Data analysis

Analysis of the data from disease assay:

1. Enter the number of bacteria from dilutions (we usually choose the dilutions with 10-100 colonies for calculation) with corresponding dilution factor in Microsoft Excel.
2. Average the two replicates of each sample. Multiply this average by a multitude of dilution factor to get the total number of colonies (colony forming units, CFU) in the sample. For example, if we get an average of 56 and the dilution factor is 10^{-5} , then the total number of colonies is 56×10^5 CFU.
3. Calculate the total leaf area. The area of one leaf disc of 7.5 mm diameter is equal to π multiplied by 0.375 squared, so the area is 0.441786 cm^2 ; there are four leaf discs in one sample, so the total leaf area is 1.767 cm^2 .
4. Divide the total number of bacteria of each sample by the leaf area to get the number of colonies per unit area. This value represents the disease susceptibility of the plant, and the higher the value, the more susceptible the plant is. For example, the number of colonies per unit area of the above sample from step1 is $56 \times 10^5 / 1.767 = 3169213 \text{ CFU/cm}^2$.
5. Take the logarithm of base 10 of the number of colonies per unit area of each sample. For example, the above value from step 4 is $\log_{10} 3169213 = 6.5 \log_{10} (\text{CFU/cm}^2)$.
6. Enter all values from all samples in GraphPad, and calculate the average and standard deviation (SD) of 3 biological replicates, draw the figure and determine statistical significance using two-way ANOVA with Tukey's test by GraphPad (Figure 2).

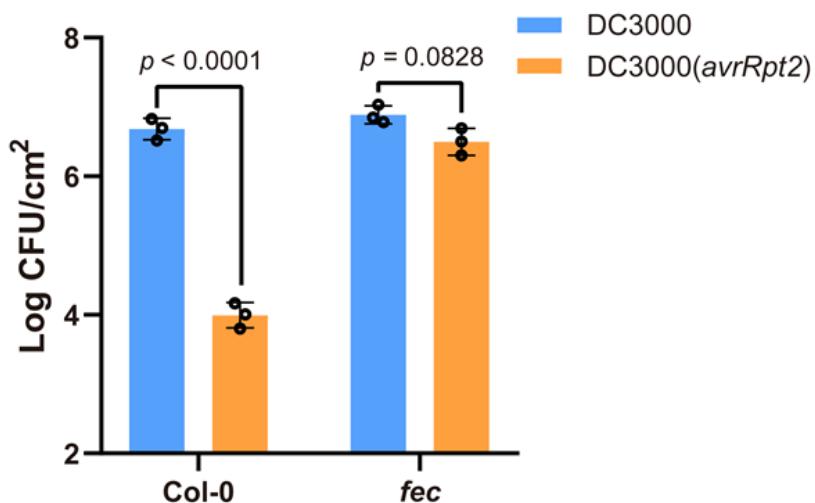


Figure 2. Example graph of disease infection assay.

The above graph was generated from one biological replicate of disease assay from Yuan *et al.* (2021). *Pst* DC3000 (*avrRpt2*) bacteria were infiltrated into *Arabidopsis* leaves at OD₆₀₀ of 0.002 and populations were determined at 3 dpi (mean \pm S.D.; n = 3 biologically independent samples). Data were analyzed using two-way ANOVA with Tukey's test.

Notes

1. We found that plant health status is one of the key determinants of disease phenotypes. Stressed or sub-optimal plants often give inconsistent or even opposite results. Abiotic conditions (such as light intensity/photoperiod,

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- temperature, and water/soil wetness) in the chamber influence the health and basal defense level of plants. It's important to use healthy plants that are grown under optimized conditions, and have a minimized or low level of basal defense for pathogen-related assays. We usually use 10 or 12 h light, keep the watering frequency/amount (not watering too much each time and avoid leaving standing water in the tray), and use plants at appropriate ages (usually around 4 weeks, as older plants sometimes tend to get anthocyanin accumulation and higher basal defense). Plants that look dark green or purple in the center of the rosettes are usually not used in our assays. Plants that are infected by insects or fungi also can not be used.
2. Leaf age may affect hormonal level and plant immunity. Leaves of a similar age should be used in these assays. We usually use 3 leaves that are middle-aged and fully expanded from each plant. Bacterial dose and sampling time (e.g., day 2 instead of day 3 or 4) can be adjusted in experiments, depending on plant condition, and bacterial strains used.
 3. Environmental factors such as light, temperature and humidity affect disease development and severity level. We found disease development to be very sensitive to air humidity, so we usually cover the inoculated plants with a transparent plastic dome (fully covered) to keep relatively high air humidity inside (Figure 1D, above 90% relative humidity) for 3-4 days.
 4. For hypersensitive response assays, wounding and leaf-age seem to have a major effect on cell death rate. We observed that mechanical wounding during syringe infiltration dramatically accelerates tissue collapse. Thus, care should be taken during injection with the syringe. We also found that younger leaves are usually associated with faster cell death, so leaves at similar age should be chosen for a fair comparison, or to observe a less obvious HR phenotype.

Recipes

1. Luria-Marine (LM) medium

Tryptone 10 g/L
Yeast Extract Powder 6 g/L
 KH_2PO_4 1.5 g/L
NaCl 0.6 g/L
 MgSO_4 0.35 g/L

Dissolve the ingredients in distilled water, adjust the pH to 7 with 10 N sodium hydroxide (NaOH) solution, and autoclave the solution.

For LM solid medium, add 15 g/L agar powder in liquid medium before autoclaving.

2. Rifampicin stock solution (1,000×)

Dissolve 2.5 g of the rifampicin powder in 50 mL dimethyl sulfoxide (DMSO) to make a 50 g/L stock solution, mix well by vortex, aliquot the solution into 1.5 mL Eppendorf tubes, and store at -20°C.

3. Spectinomycin stock solution (1,000×)

Dissolve 2.5 g of the spectinomycin powder in 50 mL sterilized water to make 50 g/L stock solution, mix well by vortex, sterilize the solution by filtering through a 0.22 μm Millex-GP Syringe Filter, aliquot into 1.5 mL Eppendorf tubes, and store at -20°C.

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Competing interests

The authors declare no competing interests

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