

Special Issue

Protocols for Coronavirus/COVID-19 Research



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Introduction

With the spread of the COVID-19 (SARS-CoV-2) pandemic across the world, sharing accurate and reproducible methods that can be readily available to the biomedical community became crucial. Therefore, *Bio-protocol* decided to dedicate a special issue to protocols used in Coronavirus/COVID-19 research in May 2020. By November 2021, we have published 19 high quality protocols used in basic science and clinical research, including diagnostic methods for COVID-19. Additionally, 9 protocols published before May 2020, used in tangential research on other coronaviruses, are also included in this special issue.

More new protocols will be welcomed to be included in this special issue, and we hope it will become a comprehensive collection of detailed protocols focused on Coronavirus/COVID-19 research. Typically, we only accept protocols previously used in published articles reporting original research. However, due to the rapid rate of preprint publications during the pandemic, we also include protocols used in preprint articles and preprint versions of the protocols.

About Bio-protocol journal

Bio-protocol was established in 2011 by a group of postdoctoral researchers at Stanford University. Our mission is to improve research reproducibility and method usability by publishing high-quality, peer-reviewed life science protocols. **Bio-protocol** has published over 4000 protocols contributed by over 10,000 scientists worldwide. Our editorial and reviewing boards consist primarily of postdocs and junior faculty members. These early-career life science researchers, who actively and routinely design, conduct, and supervise lab experiments, are well-positioned to assess the quality and clarity of protocols.

Bio-protocol has built tight collaborations with several high-profile research journals/publishers (**e.g.**, [Science/AAAS](#) & [eLife](#)) to advance our mutual goal of making scientific research more reproducible.

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RNA Isolation and Northern Blot Analysis

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[Abstract] The northern blot is a technique used in molecular biology research to study gene expression by detection of RNA in a sample. With northern blotting it is possible to observe particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. Here, we examine ATF3, ATF4, and GADD153 gene expression profiles by northern blot in Vero cells and H1299 cells after IBV infection. RNA was extracted in IBV (infectious bronchitis virus) infected cells and electrophoresis was used to separate the RNA sample. RNA was transferred from the electrophoresis gel to the blotting membrane by capillary transfer. Specific mRNA was detected with hybridization probes complementary to part of target sequence. The probes were prepared by RT-PCR and labeled by digoxigenin (DIG) using DIG labeling kit.

Materials and Reagents

1. Vero cells (kidney epithelial cells extracted from an African green monkey) (ATCC, catalog number: CCL-81™)

2. H1299 cells (human lung carcinoma cell line) (ATCC, catalog number: CRL-5803™)

3. The egg-adapted Beaudette strain of IBV (ATCC, catalog number: VR-22)

4. Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gibco®, catalog number: 11960-044)

Note: It contains more vitamins and more glucose, as well as iron and is suitable for most types of cells.

5. Roswell Park Memorial Institute medium (RPMI) 1640 (Life Technologies, Gibco®, catalog number: 21870-076)

Note: This medium contains a great deal of phosphate and is formulated for use in a 5% carbon dioxide atmosphere.

6. Trypsin/EDTA (Life Technologies, Gibco®, catalog number: 25200-072)

7. TRIzol reagent (Life Technologies, Gibco®, catalog number: 15596-018)

8. Chloroform (Thermo Fisher Scientific, catalog number: C4960/17)

9. Isopropanol (Thermo Fisher Scientific, catalog number: P7507/17)

10. Ethanol (Merck KGaA, catalog number: 1.00983.2500)

11. RNase free water

12. Reverse transcriptase AMV (Roche Diagnostics, catalog number: 10109118001)

13. Oligo (dT) (1st Base Biochemicals)
14. RNasin® ribonuclease inhibitor (Promega Corporation, catalog number: N2511)
15. Primers (1st Base Biochemicals)
16. DIG labeling kit (Roche, catalog number: 11175025910)
17. RNA loading buffer (New England Biolabs, catalog number: B0363S)
18. Agarose (1st Base Biochemicals, catalog number: BIO-100-500G)
19. Formaldehyde (Thermo Fisher Scientific, catalog number: F75P1GAL))
20. Ethidium bromide (Bio-Rad, catalog number: 1610433)
21. Hybond™-N⁺ membrane (Amersham Biosciences, catalog number: RPN303B)
22. DIG Wash and Block Buffer Set (Roche Diagnostics, catalog number: 11585762001)
23. DIG easy Hyb (Roche Diagnostics, catalog number: 11603558001)
24. Anti-digoxigenin-AP fab fragments (Roche Diagnostics, catalog number: 11093274910)
25. CDP-Star (Roche Diagnostics, catalog number: 12041677001)
26. Amersham hyperfilm ECL (Amersham Biosciences, catalog number: 28906837)
27. 70% RNase-free ethanol
28. Tris(hydroxymethyl)aminomethane (Tris base) (Promega Corporation, catalog number: H5135)
29. Acetic acid (Glacial) (Merck KGaA, catalog number: 1.00063.2500)
30. 3-(4-morpholino) propane sulfonic acid (MOPS) (Thermo Fisher Scientific, catalog number: BP308-500)
31. Sodium acetate·3H₂O (Thermo Fisher Scientific, catalog number: S207-10)
32. Sodium Citrate (Thermo Fisher Scientific, catalog number: S25545)
33. 10× TAE Electrophoresis Buffer (1 L) (see Recipes)
34. 10× MOPS buffer (1 L) (see Recipes)
35. 1× MOPS buffer (1 L) (see Recipes)
36. 1.3% Formaldehyde Agarose gel (see Recipes)
37. 20× SSC buffer (1 L) (see Recipes)
38. 2× SSC, 0.1% SDS (1 L) (see Recipes)
39. 0.1× SSC, 0.1% SDS (see Recipes)

Equipment

1. 100 mm cell culture dishes (Corning, catalog number:430167)
2. 0.2 ml thin-wall Gene-Amp PCR tube (Corning, Axygen®, catalog number: PCR-02-C)
3. Forma™ Steri-Cycle™ CO₂ Incubators (Thermo Fisher Scientific, catalog number: 201370)
4. OLYMPUS CKX31 microscope
5. Eppendorf centrifuge 5415R
6. NanoDrop (Thermo Fisher Scientific, model: ND-1000 spectrophotometer)
7. Power Pac and electrophoresis tank (Bio-Rad Laboratories)
8. Tray

9. Glass plate
10. Tissue paper
11. CL-1000, ultraviolet crosslinker (UVP)
12. Hybaid Maxi 14 Hybridization Oven (Thermo Fisher Scientific)
13. Hybridization tubes
14. Kodak Biomax cassette (Eastman Kodak Company)
15. Kodak X-OMAT 2000 processor (Eastman Kodak Company)

Procedure

A. RNA extraction

1. Cells were seeded in 100-mm-diameter dishes and infected with either 2 PFU of live IBV per cell or the same amount of UV-inactivated IBV (UV-IBV) at 37°C. Excess virus in the medium was removed by replacing with fresh medium at 1 h post-infection.
2. The IBV-infected cells were incubated at 37°C in 5% CO₂.
3. At the indicated time points (0, 2, 4, 8, 12, 16, 20, 24, 28 h post-infection), cells were rinsed with 10 ml Phosphate Buffered Saline (PBS) buffer once and lysed in 1 ml TRIzol for 5 min at room temperature.
4. Cell lysates were transfer into eppendorf tubes and one-fifth (volume/volume) of chloroform was added to each tube.
5. Shake tubes vigorously by hand for 15 sec and incubated for 3 min at room temperature, then centrifuged at 12,000 × g for 15 min at 4°C.
6. The upper aqueous phase was transfer into a new tube and mixed with 1:1 (volume/volume) of 100% isopropanol, and then incubated for 10 min at room temperature.
7. RNA was precipitated by centrifugation at 12,000 × g for 10 min at 4°C.
8. RNA pellet was washed with 1 ml 70% RNase-free ethanol once and spin down by 7,500 × g for 5 min.
9. The RNA pellets are air-dried and dissolved in 100 µl RNase-free H₂O by incubating at 65°C for 15 min.
10. RNA concentration and purity were determined by NanoDrop.
11. The RNAs were stored at -80°C for further use.

B. Probe preparation

1. Northern blot probes were obtained by RT-PCR and labeled by digoxigenin (DIG) using DIG labeling kit described as follow steps.
2. 2 µg of total RNA is added to 2 µl of 10 pmoles of an oligo (dT) in a sterile 0.2 ml thin-wall GeneAmp PCR tube of a final volume of 10.5 µl.
3. After denaturation at 65°C for 10 min, the tubes are cooled on ice immediately.

4. The denatured RNA-primer mixture is then added to a final volume of 20 μ l reaction mixture containing 10 mM of dNTPs, 20 units of Rnasin® ribonuclease inhibitor, 1 \times Expand™ reverse transcriptase buffer and 50 units of reverse transcriptase.
5. The first strand cDNA is synthesized at 43°C for 1 h, and reaction can be terminated by heating at 65°C for 10 min (optional).
6. Amplification of cDNA was achieved by polymerase chain reaction (PCR) in a 25 or 50 μ l reactions containing of appropriate primer pairs and PFU polymerase using the DIG labeling kit according to the manufacturer's manual.
7. Primers used for human ATF4 were 5'-CCGTCCCCAACCTTACGATC-3' (forward) and 5'-ACTATCCTCAACTAGGGGAC-3' (reverse). Primers used for human ATF3 were 5'-GGTAGGACTCTCCACTCAA-3 (forward) and 5'-AGACAGTAGGCCAGCGTCCTT-3' (reverse). Primers used for human GADD153 were 5'-GATTCCAGTCAGAGCTCCCT3' (forward) and 5'-GTAGTGTGGCCAAGTGGGG-3' (reverse). Prepare a 10x concentration solution of each respective PCR primer.
8. Add the following reagents in a 0.2 ml reaction tube on ice, in the following order: ddH₂O 32.25 μ l, PCR buffer 5 μ l, PCR DIG labeling mix 5 μ l, forward primer 5 μ l, reverse primer 5 μ l, enzyme mix 0.75 μ l, template cDNA 2 μ l, final volume 50 μ l. Vortex the mixture and centrifuge briefly.
9. Place the sample in a thermal block cycler and perform PCR in following condition: initial denature at 95°C for 2 min, denature at 95°C for 10 sec, anneal at 60°C for 30 sec, and elongate at 72°C for 2 min, repeat denaturation, annealing, and elongation for 30 cycles, finally elongate at 72°C for 7 min.
10. Run a portion of each PCR reaction on an agarose mini gel and then stain the gel with ethidium bromide and examine the PCR products under UV.

C. Northern blot

1. To analyze RNA expression by Northern blot, 30 μ g of RNA from each sample preparation was mixed with RNA loading buffer and load on wells in 1.3% agarose formaldehyde gel (see Recipes).
2. Run the gel with 3-4 V/cm in RNase free gel boxes for 4 h until the RNAs are well separated.
3. Stain the gel briefly in 0.25-0.5 μ g/ml ethidium bromide and examine the gel under UV light.
4. Rinse gels for 2 \times 15 min in 20 \times SSC and RNA on the gel were transferred onto a Hybond™-N⁺ membrane by capillary transfer with 20 \times SSC overnight at room temperature.
5. Fix the RNA to the membrane by UV-crosslinking. The energy used is 20,000 μ Joules/cm² at 245 nm.
6. After the UV-crosslinking, rinse the membranes briefly in ddH₂O and allow to air-dry.
7. Prehybridize membranes with DIG easy Hyb for 30 min with gentle agitation at 68°C.
8. Denature DIG-labeled RNA probes by boiling for 5 min and rapidly cooling in ice, and add the denatured probes (25 ng/ml) to 10 ml prewarmed DIG Easy Hyb.

9. 10 ml probe/hybridization mixtures were added to membranes and incubated for 6 h at 68°C with gentle agitation.
10. After hybridization, membranes were washed with 2× SSC, 0.1% SDS for 2 × 5 min at 25°C under constant agitation, and then washed with 0.1× SSC, 0.1% SDS for 2 × 15 min at 68°C under constant agitation.

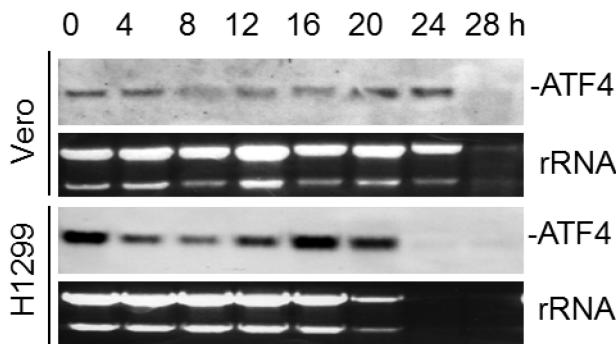


Figure 1. Northern blot analysis of ATF4 mRNA in IBV-infected cells. Vero and H1299 cells were infected with IBV (MOI~1) and harvested at indicated time points. Total RNA was isolated and subjected to Northern blot using an ATF4 probe. Ethidium bromide staining of 28S rRNA and 18S rRNA is shown as a loading control. Band intensities of ATF4 were determined and normalized to rRNA.

11. The membranes were then rinsed briefly (5 min) in washing buffer and was blocked in blocking buffer for 30 min.
12. After blocking, membranes were incubated with DIG antibody (Dilute anti-DIG-AP 1:10,000 in blocking buffer) for 30 min, washed 2 × 15 min in washing buffer and equilibrated 3 min in detection buffer.
13. The signal was detected with CDP-Star according to the manufacturer's instructions (see Figures 1-3).

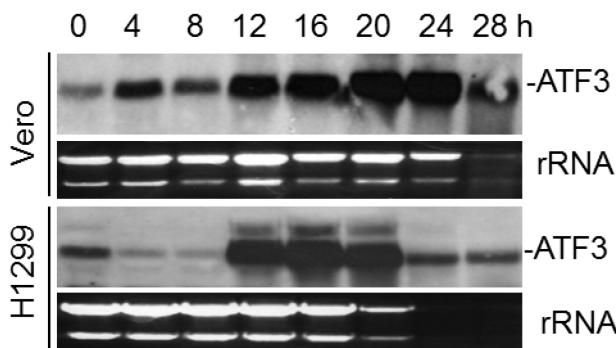


Figure 2. Northern blot analysis of ATF3 mRNA in IBV-infected cells. Vero and H1299 cells were infected as in Figure 1. RNA extraction and Northern blot was performed as in Figure 1 using ATF3 probe.

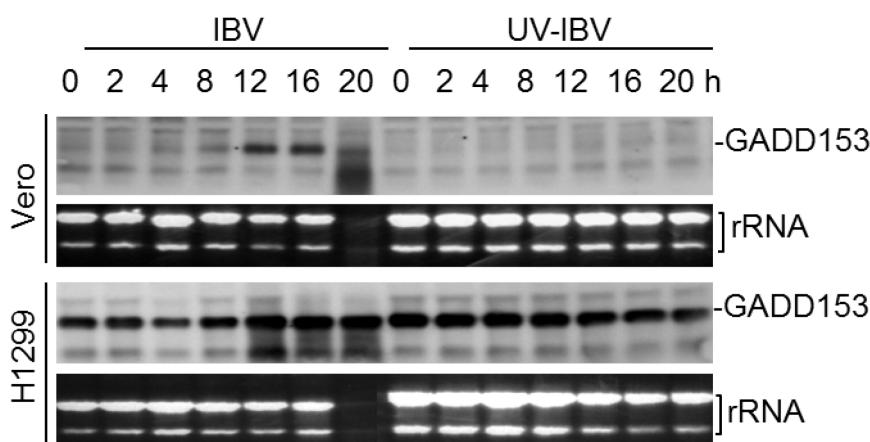


Figure 3. Northern blot analysis of GADD153 at the mRNA level in IBV-infected cells. Vero and H1299 cells were infected with IBV (MOI~1) or incubated with UV-IBV and harvested at indicated time points. RNA extraction and Northern blot was performed as in Figure 1 using GADD153 probe.

Recipes

1. 10× TAE Electrophoresis Buffer (1 L)
48.4 g Tris (hydroxymethyl) aminomethane (Tris base)
11.4 ml 17.4 M glacial acetic acid
3.7 g EDTA, disodium salt
ddH₂O
2. 10× MOPS buffer (1 L)
83.7 g 3-(N-morpholino) propanesulfonic acid (MOPS)
13.61 g Sodium acetate·3H₂O
3.7 g EDTA
ddH₂O
3. 1× MOPS buffer (1 L)
100 ml 10× MOPS buffer
20 ml 37%-formaldehyde
880 ml ddH₂O
4. 1.3% Formaldehyde Agarose gel
1.3 g agarose
10 ml 10× Formaldehyde Agarose gel buffer
Add RNase-free water to 100 ml
Heat the mixture to melt agarose
Cool to 65°C in a water bath.
Add 1.8 ml of 37% (12.3 M) formaldehyde (*toxic*) and 1 µl of a 10 mg/ml ethidium Bromide stock solution

Mix thoroughly and pour onto gel support

Prior to running the gel, equilibrate in 1× Formaldehyde Agarose gel running buffer for at least 30 min

5. 20× SSC buffer (1 L)

175.3 g of NaCl

88.2 g of Sodium Citrate

ddH₂O

Adjust the pH to 7.0 with a few drops of 14 N solution of HCl

Sterilized by autoclaving

6. 2× SSC, 0.1% SDS (1 L)

100 ml 20× SSC buffer

10 ml 10%SDS

890 ml ddH₂O

7. 0.1× SSC, 0.1% SDS

10 ml 20× SSC buffer

10 ml 10%SDS

980 ml ddH₂O

Acknowledgments

This protocol was adapted from the previous publication Liao *et al.* (2013). This work was partially supported by a Competitive Research Programme (CRP) grant (R-154-000-529-281) from the National Research Foundation, Singapore.

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RNA-Affinity Chromatography

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[Abstract] RNA-affinity chromatography assays are used to identify proteins binding specific RNA sequences. These proteins represent potential factors contributing to the function of RNA molecules. In our lab, we have used this protocol to identify proteins binding sequence motifs involved in replication and transcription of positive strand RNA viruses. The assay described in this protocol consists on the immobilization of 5'-biotinylated RNA oligonucleotides (30-40 nt) on a streptavidin-conjugated, paramagnetic solid matrix. Then, cytoplasmic protein extracts pre-cleared on the solid matrix to decrease nonspecific binding, were incubated with the immobilized RNA molecules in the presence of a nonspecific competitor. RNA-protein complexes immobilized on the paramagnetic solid matrix were isolated using a magnet and the bound proteins were separated by polyacrylamide gel electrophoresis for proteomic analysis.

Materials and Reagents

1. 5'-biotinylated RNAs (Sigma-Aldrich)
2. Streptavidin conjugated solid matrix (Dynabeads M-280 Streptavidin) (Life Technologies, Invitrogen™, catalog number: 11205D)
3. Protease inhibitor (Complete Protease Inhibitor Cocktail Tablets) (Roche, catalog number: 1697498)
4. 10% glycerol
5. Non-specific competitor tRNA (Baker yeast) (Sigma-Aldrich, catalog number: R8759)
6. NuPAGE LDS sample buffer (Life Technologies, Invitrogen™)
7. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D9779)
8. Bis-Tris-Gel (Life Technologies, Invitrogen™)
9. NuPAGE MOPS SDS running buffer (Life Technologies, Invitrogen™)
10. Coomassie Simply Blue Safe Stain (Life Technologies, Invitrogen™, catalog number: LC6060)
11. Diethylpyrocarbonate (DEPC) treated water
12. RNase inhibitor (Rnasin) (Promega Corporation, catalog number: N2611)
13. IGEPAL CA-630 (NP-40 substitute) (Sigma-Aldrich, catalog number: I3021)
14. KCl
15. Glycerol
16. NP-40

17. EDTA
18. Extraction buffer (see Recipes)
19. H-BW solution (see Recipes)
20. BW solution (see Recipes)

Equipment

1. 150 mm plates
2. Protein gel cassettes
3. Orbital Shaker (J.P. Selecta, catalog number: Orbit 3000445)
4. Magnetic particle concentrator for microcentrifuge tubes (Dynal Biotech, Dynal MPC-S 120.20)

Software

1. Image Lab V3.0 (Bio-Rad Laboratories)

Procedure

1. The capture of proteins binding specific RNA was performed using 5'-biotinylated RNAs linked to a streptavidin conjugated solid matrix.
2. Proteins were extracted from transmissible gastroenteritis virus (TGEV) infected human Huh7 cells using an extraction buffer containing 10% Igepal (NP-40) detergent. The cell lysate was then centrifuged to pellet the nuclei and save the cytoplasmic extract. Briefly, Huh7 cells grown on 150 mm plates were infected with TGEV virus. At 48 h post infection, cells from two plates were harvested and resuspended with 500 µl extraction buffer without Igepal (NP-40) on ice. Cellular extracts were incubated for 15 min on ice and then, Igepal (NP-40) detergent was added to the cell suspension to a final concentration of 10%. Extracts were mixed by vortexing, incubated for 10 additional minutes on ice and centrifuged for 2 min at maximum speed to recover the supernatant. Protein extracts may be stored at -80°C with 10% glycerol.
3. 60 µl of streptavidin-conjugated solid matrix (10 µg/µl) were used per RNA binding assay. Before binding to RNA, the solid matrix was washed twice with 360 µl of solution H-BW. All washes were performed by inverting the tube. No incubation time was required. After washing, the solid matrix was separated from the supernatant using a magnet. Leave the tubes in the magnet for 1-2 min.
4. Pre-clearing protein extracts on solid matrix not bound to RNA. Protein extracts diluted in H-BW solution (500 µg of total protein per RNA binding assay), were precleared three times by incubating with 60 µl of solid matrix in an orbital shaker at 12 rpm, for at least 5 h each time at 4°C. Separate the solid matrix using a magnet. Leave the tubes in the magnet for 1-2 min. The solid matrix was discarded after each pre-clearing incubation and the supernatant was

transferred to a new tube containing 60 µl of new solid matrix. After the third preclearing, the supernatant was preserved for RNA-binding (step 7).

5. RNA-immobilization on the streptavidin solid matrix. For each RNA binding assay, 60 µl of solid matrix were used. Previously, the solid matrix was washed twice with 60 µl of BW solution as a minimum volume. Then, the streptavidin matrix was incubated with the biotinylated RNA (8 µg) in 60 µl of BW solution for 30 min at RT.
6. Immobilized RNAs on the solid matrix were washed twice with 60 µl of H-BW solution as a minimum volume. Remove the wash solution after placing the tubes in a magnet.
7. RNA-protein binding. Add to the immobilized RNAs, 500 µg of precleared protein extract (from step 4) resuspended in H-BW solution and different amounts of non-specific competitor tRNA (0.5 or 1.25 µg tRNAs /µg protein). Incubate the mixture overnight in an orbital shaker at 4 °C and a speed of 12 rpm. The total volume of the sample was 180 µl (three times the solid matrix volume) consisting of 120 µl of precleared protein extract, 25 µl of tRNAs and 35 µl of H-BW. All the solutions were prepared in DEPC-water in the presence of 0.4 U/µl of RNase inhibitor to minimize RNA degradation.
8. Place the tubes in a magnet and remove the supernatant containing non-bound proteins. Wash three times with 120 µl H-BW solution.
9. Elute the proteins bound to immobilized RNAs with NuPAGE LDS sample buffer supplemented with DTT 100 mM for 10 min at RT.
10. Proteins bound to RNAs were resolved in NuPAGE 4-12% Bis-Tris-Gel by electrophoresis with NuPAGE MOPS SDS running buffer. Finally gels were stained with Coomassie Simply Blue Safe Stain. Images were taken with Image Lab V3.0.

Recipes

1. Extraction buffer

2.5 mM HEPES (pH 7.9)

2.5 mM KCl

25 µM EDTA

0.25 mM DTT

Protease inhibitor: One tablet diluted in 25 ml extraction buffer.

Igepal is added (if needed) to a final concentration of 10%.

2. H-BW solution

50 mM HEPES (pH 7.9)

150 mM KCl

5% glycerol

0.01% NP-40

3. BW solution

5 mM Tris HCl (pH 7.5)

1 mM EDTA

1 M NaCl

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Affinity Pulldown of Biotinylated RNA for Detection of Protein-RNA Complexes

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[Abstract] RNA-binding proteins (RBPs) have recently emerged as crucial players in the regulation of gene expression. The interactions of RBPs with target mRNAs control the levels of gene products by altering different regulatory steps, including pre-mRNA splicing and maturation, nuclear mRNA export, and mRNA stability and translation (Glisovic *et al.*, 2008). There are several methodologies available today to identify RNAs bound to specific RBPs; some detect only recombinant molecules *in vitro*, others detect recombinant and endogenous molecules, while others detect only endogenous molecules. Examples include systematic evolution of ligands by exponential enrichment (SELEX), biotinylated RNA pulldown assay, RNA immunoprecipitation (RIP) assay, electrophoretic mobility shift assay (EMSA), RNA footprinting analysis, and various UV crosslinking and immunoprecipitation (CLIP) methods such as CLIP, PAR-CLIP, and iCLIP (Popova *et al.*, 2015). Here, we describe a simple and informative method to study and identify the RNA region of interaction between an RBP and its target transcript (Panda *et al.*, 2014 and 2016). Its reproducibility and ease of use make this protocol a fast and useful method to identify interactions between RBPs and specific RNAs.

Keywords: Tagged RNA, RNA-binding proteins, Ribonucleoprotein complex, Biotin pulldown, *In vitro* transcription

[Background] RNA-protein interactions critically influence gene expression patterns. The identification of these ribonucleoprotein (RNP) complexes is essential for understanding the regulatory mechanisms governed by RNA-binding proteins (RBPs). Recently, extensive efforts have led to the development of methods for systematic analysis of RNA-protein interactions. Highly informative methods to identify RNP complexes include a number of different types of RNP immunoprecipitation (IP) analyses. RIP methods involve RNP IP without crosslinking, while CLIP methods involve crosslinking of the RNP before IP. While RIP is fast, inexpensive, and capable of assessing many endogenous RBPs and RNAs, it does not typically permit the identification of the precise RNA region that interacts with the RBP. CLIP analysis (including its variant forms HITS-CLIP, PAR-CLIP, and iCLIP) does allow the discovery of the precise RNA sequences that interact with an RBP, as it includes an RNase step that digests all unprotected RNA and yields the RNA bound to the RBP. However, CLIP analysis is costly, time-consuming, and technically challenging (Panda *et al.*, 2016). Therefore, alternatives to testing the binding of endogenous proteins to RNAs of interest are needed.

The biotinylated RNA-pulldown method described here theoretically works for all RBPs, as this assay

is performed in a cell-free system. The method involves the *in vitro* synthesis of RNAs of interest in the presence of biotinylated CTP; the RNA tagged in this manner is then incubated with a cell-free system to allow RBPs to recognize RNA regions to which it has affinity, while regions without affinity do not interact with RBPs. After the binding is complete, the biotinylated RNA is pulled down using streptavidin-coated beads and the RBPs are typically detected by Western blot analysis. This method can be used to map the RNA sequence with which the RBP interacts if the user tests progressively smaller RNA fragments in a systematic fashion, as described here. Furthermore, this method allows for the identification of all of the proteins that interact with the RNA of interest if the biotin-RNA pulldown is followed by mass spectroscopy. In summary, this approach can successfully identify the interaction of an endogenous (or recombinant) RBP with *in vitro*-synthesized RNAs of interest.

Materials and Reagents

1. ThermoGrid™ rigid strip 0.2-ml PCR tubes (Denville Scientific, catalog number: C18064 [1000859])
2. Posi-Click 1.7-ml microcentrifuge tube (Denville Scientific, catalog number: C2171)
3. 1.5-ml tubes
4. 10-cm dishes
5. NucAway spin columns (Thermo Fisher Scientific, Invitrogen™, catalog number: AM10070)
6. Disposable cuvettes, 1.5-ml (Stockwell Scientific, catalog number: 2410)
7. Nuclease-free water (Thermo Fisher Scientific, Ambion™, catalog number: AM9930)
8. cDNA prepared from total RNA
9. DreamTaq DNA polymerase (5 U/μl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EP0701)
10. dNTP mix (10 mM each) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0193)
11. Agarose LE (Denville Scientific, catalog number: CA3510-8)
12. QIAquick Gel Extraction Kit (50) (QIAGEN, catalog number: 28704)
13. MEGAshortscript™ T7 Kit with manual (for RNA shorter than 0.5 Kb) (Thermo Fisher Scientific, Invitrogen™, catalog number: AM1354)
14. RiboLock RNase inhibitor (40 U/μl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EO0381)
15. Biotin-14-CTP (Thermo Fisher Scientific, Invitrogen™, catalog number: 19519-016)
16. MEGAscript® T7 Transcription Kit (*for RNA longer than 0.5 Kb) (Thermo Fisher Scientific, Invitrogen™, catalog number: AM1333)
17. Novex® TBE-urea gels, 6% (Thermo Fisher Scientific, Invitrogen™, catalog number: EC6865BOX)
18. 1x TBE buffer
19. Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14040-133)

20. cOmplete protease inhibitor cocktail (Sigma-Aldrich, catalog number: 11697498001)
21. 2x Laemmli sample buffer (Bio-Rad Laboratories, catalog number: 1610737)
22. Dynabeads® M-280 streptavidin (Thermo Fisher Scientific, Invitrogen™, catalog number: 11205D)
23. 2-mercaptoethanol (β -mercaptoethanol/BME)
24. 10× Tris/glycine/SDS running buffer (Bio-Rad Laboratories, catalog number: 1610732)
25. 4-20% Mini-PROTEAN® TGX Stain-Free™ protein gels (Bio-Rad Laboratories, catalog number: 4568094)
26. Ethidium bromide solution (Sigma-Aldrich, catalog number: E1510)
27. Spectra™ multicolor broad range protein ladder (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 26634)
28. Bio-Rad protein assay dye reagent concentrate (Bradford reagent) (Bio-Rad Laboratories, catalog number: 500-0006)
29. Tris-HCl (pH 8.0)
30. KCl
31. MgCl₂
32. Nonidet P-40
33. EDTA
34. NaCl
35. Triton X-100
36. Polysome extraction buffer (PEB) (see Recipes)
37. 2× Tris, EDTA, NaCl, Triton (TENT) buffer (see Recipes)
38. 1× TENT (see Recipes)

Equipment

1. PCR strip tube rotor, mini centrifuge C1201 (Denville Scientific, catalog number: C1201-S [1000806])
2. Veriti® 96-Well thermal cycler (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4375786)
3. Ultraviolet transilluminator
4. NanoDrop One spectrophotometer (Thermo Fisher Scientific, Thermo Scientific, catalog number: ND-ONE-W)
5. Eppendorf Thermomixer® R (Eppendorf, catalog number: 022670581)
6. Incubator
7. Vortexer
8. Cell scrapers
9. Refrigerated centrifuge (Eppendorf, model: 5430R)

10. SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, catalog number: 1702525) or other spectrophotometer with 595 nm wavelength
11. MagneSphere(R) stand (Promega, catalog number: Z5342)
12. Trans-Blot® Turbo™ transfer starter system (Bio-Rad Laboratories, catalog number: 1704155)
13. Mini-PROTEAN® tetra vertical electrophoresis cell (Bio-Rad Laboratories, catalog number: 1658004)

Procedure

A. Primer design and template generation for *in vitro* transcription

1. The forward and reverse primers with a length of 20-25 nt and melting temperature of ~60°C are designed using Primer 3 online tool (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Untergasser et al., 2012) (Note 1).
2. As shown in Figure 1, add the T7 RNA polymerase promoter sequence (T7) [5'AGTAATACGACTCACTATAGGG] (red lines) upstream of the actual forward primer sequence (Figure 1).
3. Dissolve the primers in nuclease-free water to a final concentration of 100 µM, then prepare a primer mix of forward and reverse primers at a final concentration of 1 µM each in nuclease-free water (e.g., 10 µl of each primer from the 100 µM stock into 980 µl of nuclease-free water and mix well).
4. For one PCR reaction of 40 µl in a 0.2-ml PCR tube, add 4 µl of 10× DreamTaq DNA polymerase buffer, 10 µl of 1 µM primer mix, 1 µl DreamTaq DNA polymerase, 1 µl of 10 mM dNTP mix, 0.2 µl (~2 ng) cDNA, and water to make the final volume 40 µl. (The cDNA was prepared from total RNA as described previously [Panda et al., 2016].)
5. Mix the reaction by vortexing for 5 sec and centrifuge the PCR tube strip for 30 sec using minicentrifuge to settle the reactions at the bottom of the wells.
6. Set up the Veriti® 96-Well Thermal Cycler program as follows: 5 min at 95°C and 40 cycles of 5 sec at 95°C and 60 sec at 60°C.
7. Resolve the PCR products on an ethidium bromide stained 2% agarose gel for 1 h at 100 volts and visualize with an ultraviolet transilluminator.
8. Cut the desired bands from the gel. Purify the PCR products corresponding to each fragment using the QIAquick Gel Extraction Kit and dissolve in elution buffer provided with the kit.
9. Measure the concentration of the PCR products/templates with a NanoDrop spectrophotometer.
10. The PCR product (T7 DNA template) can be stored at -20°C or -80°C, or used immediately for *in vitro* transcription.

B. *In vitro* transcription of biotinylated RNA fragments

1. The biotinylated RNA can be prepared using the MEGAshortscript™ T7 Kit following the manufacturer's protocol.

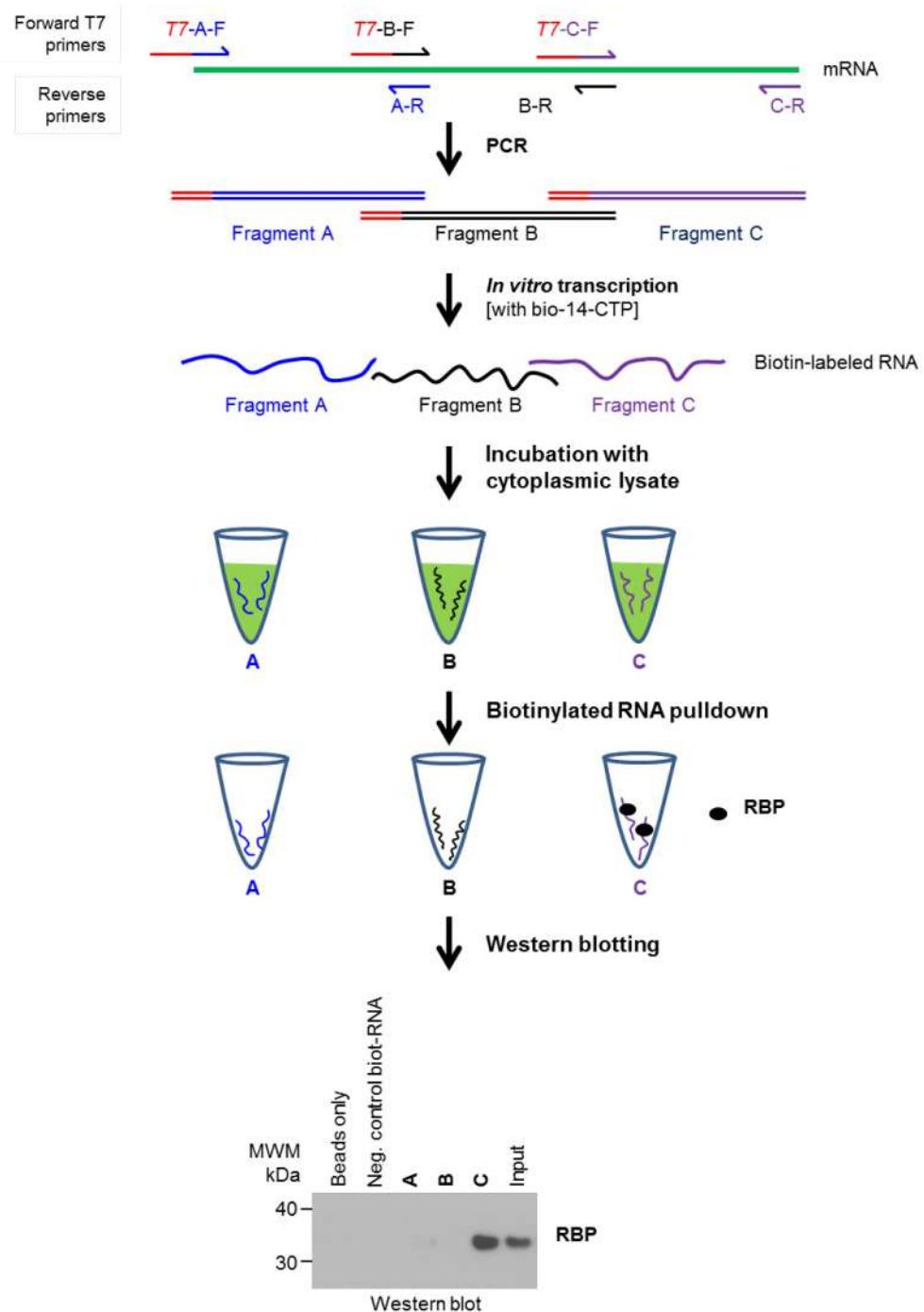


Figure 1. Schematic of biotinylated RNA pulldown assay

2. *In vitro* transcription reaction for each DNA template can be prepared in a 1.7-ml PCR tube containing 2 μ l of 10 \times MEGAshortscriptTM T7 buffer, 2 μ l of MEGAshortscriptTM T7 enzyme mix, 1 μ l of RiboLock, 1 μ l of 75 mM rATP, 1 μ l of 75 mM rUTP, 1 μ l of 75 mM rGTP, 0.8 μ l of 75 mM rCTP, 1.5 μ l of 10 mM Biotin-14-CTP, 100 ng of T7 DNA template and water to make the final volume 20 μ l. (For longer RNA fragments [> 500 nt] the user should employ the MEGAscriptTM T7 Kit for *in vitro* RNA preparation) (Notes 5 and 6).

3. Mix and centrifuge for ~5 sec at $500 \times g$ to settle the reaction mixture at the bottom of the tube.
4. Incubate the reaction at 37°C for 4 h in a Thermomixer (Note 2).
5. Add 1 μl of DNase provided in the kit and mix well followed by incubation for 15 min at 37°C .
6. Store the samples at -20°C or -80°C or proceed immediately to RNA purification.

C. Purification and quality check of *in vitro*-transcribed biotinylated RNA fragments

1. Dilute the entire 20 μl *in vitro* transcribed RNA by adding 50 μl of nuclease-free water and keep on ice.
2. Use one column (NucAway Spin Columns Kit) for each RNA fragment.
3. Tap the powders to the bottom of columns and add 650 μl of nuclease-free water followed by vortexing at the highest speed for 10 sec.
4. Incubate the column for 15 min at room temperature followed by centrifugation for 2 min at $750 \times g$.
5. Pipet the *in vitro*-transcribed RNA onto the top of the column and put the column in a fresh 1.5-ml tube.
6. Centrifuge the column for 2 min at $750 \times g$ to collect the flow-through containing biotinylated RNA.
7. The RNA concentration can be determined using the NanoDrop spectrophotometer.
8. Store the purified samples at -20°C or -80°C until the quality of RNA is checked.
9. To check the quality of the RNA, transfer 1 μg of transcribed RNA to a fresh tube and denature for 5 min at 70°C with RNA sample preparation buffer provided in the MEGAshortscript kit.
10. Resolve on a 6% TBE-Urea gel with 1 \times TBE buffer and visualize the RNAs on an ultraviolet transilluminator after staining with ethidium bromide.
11. All RNA fragments should show single bands of the expected size.
12. Store the RNA samples at -20°C or -80°C or use in the pulldown reaction.

D. Preparation of cell lysate

1. Wash two 10-cm dishes of ~70% confluent cells of interest three times with PBS and collect them using a cell scraper. As the protein yield varies with the cell line, the user should adjust the number of cells needed to extract at least 500 μg protein for each pulldown assay.
2. Pellet the cells in a 1.7-ml tube by centrifuging for 5 min at $500 \times g$.
3. Disrupt the cell pellet by pipetting 10-20 times with 500 μl of polysome extraction buffer (PEB) containing protease and RNase inhibitors. Incubate for 10 min on ice.
4. Collect the supernatant after centrifugation at $15,000 \times g$ for 10 min at 4°C using a refrigerated centrifuge.
5. Determine protein concentrations by the Bradford protein assay following the manufacturer's instructions using disposable cuvettes and a spectrophotometer at 595 nm wavelength.
6. Store protein samples at -20°C or -80°C or use directly in pulldown reaction.

E. Pulldown of interacting RNA-binding protein

1. Prepare the pulldown reaction in 1.5-ml tube containing 500 µg of cell lysate, 1 µg of purified biotinylated RNA, 1× protease inhibitor (add fresh), 5 µl of RiboLock, 250 µl of 2× TENT buffer (Tris, EDTA, NaCl, Triton; see Recipes) and PEB in 500 µl final volume.
2. Mix the reaction by pipetting and incubate at room temperature for 30 min.
3. In the meantime, wash 50 µl streptavidin-coupled dynabeads for each reaction three times with 1× TENT buffer using the MagneSphere stand.
4. Add 50 µl washed beads to the RNA-protein mixture prepared in step E1 (Note 7).
5. Mix the beads by pipetting up and down.
6. Incubate the reaction at room temperature for another 30 min with intermittent mixing by tapping the tube every ~5 min.
7. Incubate the tube on the magnetic stand for 1 min to allow the magnetic beads to settle to one side of the tube.
8. Discard the supernatant and mix the magnetic beads with 1 ml of ice-cold 1× TENT buffer.
9. Repeat steps E7 and E8 3 more times.
10. Add 40 µl of 1× Laemmli sample buffer supplemented with β-mercaptoethanol to the magnetic beads.
11. Heat the samples for 5 min at 95 °C and proceed to Western blotting.

F. Detection of interacting RNA-binding proteins (RBPs)

1. Use Western blotting techniques to detect the RBP interest in the pulldown. Briefly, load at least three lanes on a 4-20% SDS-PAGE gel: (1) 10 µg (or 2% of the protein used for pulldown) of cell lysate as input, (2) the entire sample from the pulldown assay, and (3) a pre-stained protein ladder. Resolve these samples by electrophoresis following the manufacturer's protocol.
2. Transfer the protein to a nitrocellulose membrane using a Trans-Blot transfer system following the manufacturer's instructions.
3. Use standard Western blotting techniques to detect the RBP of interest using primary antibodies recognizing that RBP (Mahmood and Yang, 2012).
4. Incubate with the appropriate secondary antibody to recognize the primary antibody and detect the signals using Enhanced Chemiluminescence (ECL).

Data analysis

As shown in Figure 1, Western blot analysis reveals the presence of the RBP in the input lysate (positive control) and in the pulldown material using biotinylated fragment C, suggesting that the sequence in fragment C includes a region with which this RBP interacts. This analysis includes negative control samples 'Beads only' and 'Neg. control biot-RNA', which do not pulldown the RBP, and shows that fragments A and B do not bind the RBP, supporting the conclusion that binding of the RBP to fragment C is specific. The experiment should be repeated at least three times to

conclude the specificity of the RNA-RBP interaction.

Notes

1. The size of the RNA fragment can be decided by the researcher, but an overlap of at least 20 bp between adjacent fragments is recommended.
2. Longer (more than 4 h) incubation of *in vitro* transcription reaction may lead to degradation of the *in vitro*-transcribed RNA.
3. As a control, use a ‘beads only’ sample without any biotinylated RNA to see if the magnetic beads alone will bind to RBP of interest in this pulldown condition.
4. Use a negative control (NC) biotinylated RNA such as *GAPDH/ACTB* that does not bind the RBP studied in order to check the specificity of interaction between the RBP and the mRNA of interest.
5. This protocol uses a ratio of 1 biotin-CTP:4 unlabeled CTP (*i.e.*, 1 in 5 CTP is biotin-labeled). The ratio between biotin-CTP and unlabeled CTP is adjusted, and the user has to be mindful of the number of Cs present in the RNA product. For long transcripts, the biotin-CTP ratio can be lowered to 1 biotin-CTP:10 unlabeled CTP.
6. Labeling the RNA at higher than 1:4 ratio of biotinylated to non-biotinylated CTP may inhibit RBP binding. The user must ensure that each RNA fragment has at least 1 or 2 biotinylated Cs in its sequence.
7. Using higher amount of biotin-labeled RNA does not help and may be detrimental to the pulldown assay, as 50 µl of streptavidin magnetic beads can bind ~100 pmol of biotin-labeled RNA.
8. Longer RNA may result in false positive pulldown result as the RBP of interest may interact with the RNA bait indirectly through another RBP.
9. Lysis of cells in PEB isolates cytoplasmic fractions effectively. Thus, this protocol is best suited for studying the interactions of cytoplasmic proteins with RNAs of interest. RBPs interacting with the RNA bait *in vitro* may or may not interact in the cells due to differential subcellular localization of RNA or protein of interest.

Recipes

1. Polysome extraction buffer (PEB) in nuclease-free water
20 mM Tris-HCl (pH 7.5)
100 mM KCl
5 mM MgCl₂
0.5% Nonidet P-40
2. 2× Tris, EDTA, NaCl, Triton (TENT) buffer in nuclease-free water
20 mM Tris-HCl (pH 8.0)

- 2 mM EDTA (pH 8.0)
500 mM NaCl
1% (v/v) Triton X-100
3. 1× TENT
Equal volumes of 2× TENT and nuclease-free water mixed together

Acknowledgments

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Biochemical Assays for MTase Activity

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[Abstract] Methyltransferase (MTase) transfers a methyl group (-CH₃) from the donor S-adenosyl-L-methionine (AdoMet or SAM) to biologically active molecules such as hormones, neurotransmitters, lipids, proteins and nucleic acids. The addition of a methyl group causes a change in the physicochemical properties of the molecules. The mRNA cap structure is essential for cell and virus. Guanine-N7-methyltransferase (N7-MTase) methylates the GpppN cap at the N7 position of guanine, resulting in cap-0 structure (m7GpppN), and Ribose 2'-O-MTase further methylates the first nucleotide of higher eukaryotic cellular and viral mRNAs at the ribose 2'-OH position to form cap-1 (m7GpppNm) structures. Here, we describe a biochemical assay to detect the activities of mRNA capping MTases.

Materials and Reagents

1. Bodicon m7G capping system (Bodicon, catalog number: CS0130)
2. S-adenosyl methionine (SAM) (involved in Bodicon m7G capping system) (Bodicon, catalog number: CS0130)
3. Bodicon Capping Enzyme (10 U/μl) (involved in Bodicon m7G capping system) (Bodicon, catalog number: CS0130)
Note: Because the sale of this kit was low, the previous companies which provide this capping kit were out of service. This capping kit was provided by a new company in China as custom-made products (contact e-mail: service@bodicon.cn, phone: +86-13628662011). In fact the similar capping kit from any other companies (such as Epicentre Biotechnologies, ScriptCap m7G capping system, catalog number: SCCE0610) is suitable for this experiment, and people can also contact with us to get the related protein or kit.
4. Inorganic pyrophosphatase (YIPP) (New England Biolabs, catalog number: M2403S)
5. S-adenosyl [³H] methionine ([³H]-SAM) (PerkinElmer, catalog number: NET155H001MC)
6. DEAE Sephadex (GE Healthcare, catalog number: 17-0170-01)
7. GTP (Thomas Scientific, catalog number: R0461)
8. RNase inhibitor (Thomas Scientific, catalog number: EO0381)
9. RNase free water
10. Phenol-chloroform (pH 4.8-5.2 for RNA only)
11. Ethanol (RNase free)
12. RNase free water

13. Sodium Dodecyl Sulfonate (SDS)
14. Ethylene Diamine Tetraacetic Acid (EDTA)
15. NH₄HCO₃
16. NaCl
17. 10× MTase Buffer (see Recipes)
18. Cap-0 cap structure (m7GpppN-RNA) (see Recipes)
19. Non-methylated Cap-0 cap structure (GpppN-RNA) (see Recipes)
20. MTase assay reaction mix (see Recipes)

Equipment

1. Bechtop
2. Water bath
3. Centrifuge
4. Liquid scintillation counter

Procedure

1. Synthesis of RNA cap structure as substrates.

The nascent RNA transcribed *in vitro* possesses a 5' triphosphate end. Two RNA capping systems are used to synthesize cap structure as the substrates of MTase.

- a. Combine 1-10 µg RNA and RNase free water up to 12.5 µl of total reaction volume.
- b. To heat to denature the *in vitro* transcribed RNA, incubate the tube at 65°C for 10 min, and transfer the tube to ice immediately.
- c. Combine the following reaction components in the order given:

Cap-0 cap structure (m7GpppN-RNA)

Heat denatured RNA	12.5 µl
10× Bodicon Capping Buffer	2 µl
10 mM GTP	2 µl
3 mM SAM	1 µl
RNase inhibitor 40 U/µl	0.5 µl
Bodicon Capping Enzyme (10 U/µl)	2 µl
Total	20 µl

Non-methylated Cap-0 cap structure (GpppN-RNA)

Heat denatured RNA	12.5 µl
10× Bodicon Capping Buffer	2 µl
10 mM GTP	2 µl
Inorganic pyrophosphatase 0.1 U/µl	1 µl

RNase inhibitor 40 U/ μ l	0.5 μ l
Bodicon Capping Enzyme (10 U/ μ l)	2 μ l
Total	20 μ l

- d. Incubate at 37°C for 2 h.
- e. Purify the RNA substrates by using phenol-chloroform extraction and ethanol precipitation methods.
2. Prepare 10× MTase Buffer. The MTase buffer may be changed depending on different interested MTases.
3. Combine the following reaction components in the order given for MTase assays:

Purified MTases	1 μ g
10× MTase Buffer	3 μ l
GpppN-RNA or m7GpppN-RNA	2 μ g
3 mM SAM	0.5 μ l
[³ H]-SAM (67.3 Ci/mmol, 0.5 μ Ci/ μ l)	1 μ l
RNase inhibitor 40 U/ μ l	1 μ l
RNase free water	up to 30 μ l
Total	30 μ l
4. Incubate the reaction at 30-37°C depending on different MTases for 1.5 h.
5. Transfer the tubes onto ice and add equal volume (30 μ l) of 0.2% SDS, 20 mM EDTA.
6. Keep the tubes on ice, add 1 ml of 10 mM NH₄HCO₃ (pH 8.5).
7. Prepare 1 ml DEAE Sephadex column and equilibrated with 10 ml 10 mM NH₄HCO₃ (pH 8.5).
8. Load the samples onto the equilibrated column.
9. Wash the column with 10 ml of 10 mM NH₄HCO₃ (pH 8.5), 100 mM NaCl.
10. Elute the samples with 1.5 ml of 10 mM NH₄HCO₃ (pH 8.5), 400 mM NaCl.
11. Add equal volume scintillation liquid, mix well by vortexing and measure the signal using Liquid scintillation counter. The counting signal of [³H], which is transformed from [³H]-SAM to RNA substrates, represents the activity of tested MTases.

Recipes

1. 10× MTase Buffer
 - 0.5 M Tris-HCl (pH 7.5 or 8.0)
 - 50 mM KCl
 - 20 mM MgCl₂
 - 20 mM DTT
2. Cap-0 cap structure (m7GpppN-RNA)
 - Heat denatured RNA 13.5 μ l
 - 10× Bodicon Capping Buffer 2 μ l

10 mM GTP	2 µl
3 mM SAM	1 µl
RNase inhibitor 40 U/µl	0.5 µl
Bodicon Capping Enzyme (10 U/µl)	2 µl
Total	20 µl
3. Non-methylated Cap-0 cap structure (GpppN-RNA)	
Heat denatured RNA	13.5 µl
10× Bodicon Capping Buffer	2 µl
10 mM GTP	2 µl
Inorganic pyrophosphatase 0.1 U/µl	1 µl
RNase inhibitor 40 U/µl	0.5 µl
Bodicon Capping Enzyme (10 U/µl)	2 µl
Total	20 µl
4. MTase assay reaction mix	
Purified MTases	1 µg
10× MTase Buffer	3 µl
GpppN-RNA or m7GpppN-RNA	2 µg
3 mM SAM	0.5 µl
[³ H]-SAM (67.3 Ci/mmol, 0.5 µCi/µl)	1 µl
RNase inhibitor 40 U/µl	1 µl
RNase free water	up to 30 µl
Total	30 µl

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Measurement of CD8 and CD4 T Cell Responses in Mouse Lungs

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[Abstract] Study of the adaptive immune response to a viral challenge in an animal model often includes analysis of the T cell response. Here we discuss in detail the methods that are used to characterize the CD8 and CD4 T cell response following viral challenge in the lung.

Materials and Reagents

1. Mice (NCI) (BALB/c or C57BL/6, 4 weeks to 18+ months)
2. Isofluorane (USP inhalation vapour, liquid) (NDC: 57319-559-06)
3. Dulbecco's modified eagle medium high glucose (DMEM) (Life Technologies, Gibco®, catalog number: 11965092)
4. Ketaset III ketamine HCl injection (USP 100 mg/ml) (DEA Schedule II Drug) (NDC: 0856-2013-01)
5. 100 mg/ml AnaSed injection xylazine (Lloyd Laboratories, NADA number: 139-236)
6. 0.9% sodium chloride irrigation (USP) (Baxter, catalog number: 2F7124)
7. 1× Dulbecco's phosphate buffered saline (DPBS) (Life Technologies, Gibco®, catalog number: 14190-144)
8. Collagenase D (Roche Diagnostics, catalog number: 11088882001)
9. DNase I (Roche Diagnostics, catalog number: 10104159001)
10. Hank's balanced salt solution (HBSS) (Life Technologies, Gibco®, catalog number: 14025)
11. L-Glutamine (200 mM) (Life Technologies, Gibco®, catalog number: 25030-081)
12. Hepes (1 M) (Life Technologies, Gibco®, catalog number: 15630-080)
13. Penicillin streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)
14. RPMI medium 1640 (Life Technologies, Gibco®, catalog number: 11875-093)
15. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250-100ML)
16. BD Golgi Plug™ (BD, catalog number: 554722)
17. Normal rat serum (Life Technologies, Invitrogen™, catalog number: 10710C)
18. FITC Anti-mouse CD8a clone 53-6.7 (0.5 mg/ml) (BD, catalog number: 553031)
19. PerCP-cyanine5.5 Anti-mouse CD4 clone RM4-5 (eBioscience, catalog number: 45-0042-82)
20. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150)
21. Sodium azide (AMRESCO, catalog number: 0639)
22. BD Cytofix/Cytoperm™ fixation and permeabilization solution (BD, catalog number: 554722)

23. BD Perm/Wash™ buffer (BD, catalog number: 554722)
24. APC Anti-mouse IFN γ clone XMG1.2 (eBioscience, catalog number: 17-7311-82)
25. Ketamine solution (see Recipes)
26. Digestion buffer (see Recipes)
27. RP10 (see Recipes)
28. FACS buffer (see Recipes)
29. Cell surface staining mixture (see Recipes)
30. Intracellular staining mixture (see Recipes)

Equipment

1. Dessicator (Narang Medical, catalog number: P37.1517P)
2. Precision glide needle (25 G × 5/8) (BD, catalog number: 305122)
3. Gauze sponges (4 × 4 inch) (Pro Advantage® by NDC, catalog number: P157118)
4. 1 ml syringe (BD, catalog number: 309659)
5. 3 ml syringe (BD, catalog number: 309657)
6. Cell strainer (70 µm nylon) (BD, catalog number: 352350)
7. Tissue culture dishes (60 × 15 mm) (BD Biosciences, Falcon®, catalog number: 353002)
8. 96 well cell culture cluster (round bottom with Lid) (Corning, Costar®, catalog number: 3799)
9. 5 ml polystyrene round bottom tube (BD Biosciences, Falcon®, catalog number: 352054)
10. Absorbent pads (Covidien, catalog number: 949)
11. 10 ml syringe (BD, catalog number: 309604)
12. 12 well cell culture cluster (flat bottom with lid) (Corning, Costar®, catalog number: 3513)
13. 1 ml graduate transfer pipette (Research Products International, catalog number: 147501-1S)
14. 15 ml screw cap tube conical (Sarstedt AG, catalog number: 62.554.002)
15. 50 ml screw cap tube conical (Sarstedt AG, catalog number: 62.547.004)
16. Biosafety hood
17. Spray bottle with 70% ethanol
18. Surgical scissors
19. Polystyrene foam
20. Pipetman p10
21. Pipetman p200
22. Pipetman p1000
23. CO₂ incubator
24. Pipet aid
25. Small metal weighing spatula
26. Tweezers
27. Vortexer
28. Shaker rotisserie

29. Refrigerated tabletop centrifuge
30. Hemocytometer
31. Flow cytometer

Procedure

A. Infection

1. Infect mice as described in [Virus Infection and Titration of SARS-CoV in Mouse Lung](#) (Fett et al., 2014).

B. Perfusion and removal of lungs

2. Euthanize mice by intraperitoneal injection of 300 µl of ketamine/xylazine solution. Secure the mouse in the palm of the hand and hold it horizontally, then insert the needle at a shallow angle tangential to the mouse and slowly dispense ketamine/xylazine into the peritoneal cavity.
3. When the mouse is fully anesthetized, immobilize it on a piece of polystyrene foam by pinning each limb with a 25 G × 5/8 needle.
4. Use a spray bottle filled with 70% ethanol to wet the fur of the mouse.
5. Cut open the abdominal and thoracic cavities with scissors by first making an incision from the lower abdominal to the throat of the animal. Opening the abdominal cavity exposes the underside of the diaphragm. Cut through the diaphragm with scissors and then remove the ribcage, fully exposing the heart and lungs.
6. Fill a 10 ml syringe with sterile PBS and attach a 25 G × 5/8 needle. Insert the needle into the left ventricle of the heart and smoothly dispense PBS into the heart. While slowly dispensing PBS into the heart, use a tweezers to dissect the right atria away from the heart allowing blood to drain from circulation. Perfuse the general circulation with 5-7 ml of PBS. Remove the needle from the left ventricle and insert it into the right ventricle to more directly perfuse the lungs with the remaining 3-5 ml of PBS. Lungs without significant disease should perfuse easily.
7. Cut the heart away from the lungs and then remove the lungs from the thoracic cavity after cutting the trachea and any remaining connective tissue.
8. Place the lungs into the well of a 12 well tissue culture plate. If multiple lungs are to be harvested place lungs in PBS, store plate on ice and then transfer to a clean dry well immediately before processing.
9. Mince the lungs into very fine pieces using a scissors.
10. Transfer minced lung with a 1 ml transfer pipette to 5 ml of digestion buffer in a 15 ml conical tube.
11. Place tubes on a shaker rotisserie and gently rotate at room temperature for 30 min.
12. Place a 70 µm cell strainer into a 60 × 15 mm tissue culture dish.
13. Transfer digestion buffer and lung tissue to the cell strainer using a 1 ml transfer pipette. Transfer some of the strained buffer into a 50 ml conical tube leaving enough liquid in the tissue culture

dish so that the lung tissue on the screen of the cell strainer stays wet. With the flat end of a plunger from a 3 ml syringe gently press and dissociate tissue through strainer using small light movements. Intermittently flush tissue with PBS to move cells through the strainer. Process tissue until there is only connective tissue remaining on the strainer and transfer the remaining digestion buffer to the 50 ml conical tube.

14. Spin down lung cells in 50 ml conical tubes for 10 min at 1,300 rpm at room temperature in a bucket tabletop centrifuge.
15. Pour off supernatant and resuspend cells in 5 ml of RP10.

C. Peptide stimulation and cell staining

16. Count cells using a hemocytometer. Normal lung cell counts can vary from 3 million to 30 million or more depending on the stage and severity of the infection.
17. Spin down cells for 5 min at 1,300 rpm.
18. Resuspend cells in RP10 at 1 million cells per 100 μ l.
19. In a 96 well round bottom plate, add 100 μ l of cells to 100 μ l of RP10 containing Golgi Plug at a concentration of 2 μ g/ml for a final concentration of 1 μ g/ml. Wells should contain designated amount of peptide. Make sure to include wells for a no peptide control. In our SARS-CoV-infected samples, we routinely stimulate cells with peptide at a concentration of 1 μ M. The SARS-CoV-specific peptides S366 (CD8; HNYKYRYL) and N353 (CD4; VNFNFNGL) are used to identify virus-specific CD8 and CD4 T cells by intracellular cytokine staining (ICS) for interferon gamma (IFN- γ). These two epitopes are H-2^d-restricted and therefore recognized in BALB/c mice.
20. Incubate cells at 37°C, 5% CO₂ for 5 h. Cells can then be stored overnight at 4°C.

D. Cell surface staining

21. Spin down cells at 1,000 rpm for 5 min at 4°C and then remove the supernatant by dumping the plate into a waste bin containing 10% bleach (when working with SARS-CoV) or rapidly flipping the plate onto paper towels.
22. Add 100 μ l of cell surface staining mixture per well. Mix thoroughly by pipeting each well up and down several times.
23. Incubate in the dark for 15 min at 4°C.
24. Add 100 μ l of FACS buffer and spin down cells at 1,300 rpm for 5 min at 4°C. Remove supernatant. Add 100 μ l of fixation and permeabilization solution; mix thoroughly by pipeting up and down several times.
25. Incubate in the dark for 30 min at 4°C.
26. If working with SARS-CoV, cells can be transferred to a new 96 well round bottom plate and then removed from the BSL3.
27. Using a pipetman, pass cells in fixation and permeabilization solution through a 70 μ m cell strainer into FACS tubes.

28. Resuspend cells in 2 ml of BD Perm/WashTM buffer, then spin down cells at 1,300 rpm for 5 min at 4°C. Remove supernatant.

E. Intracellular staining

29. Resuspend cells in 100 µl of intracellular staining mixture per sample. Mix thoroughly by lightly vortexing tubes.

30. Incubate in the dark for 30 min.

31. Resuspend cells in at least 1 ml of FACS buffer then spin down cells at 1,300 rpm for 5 min at 4°C. Remove supernatant.

32. Resuspend cells in 100 µl FACS buffer.

33. Acquire FACS data using a flow cytometer.

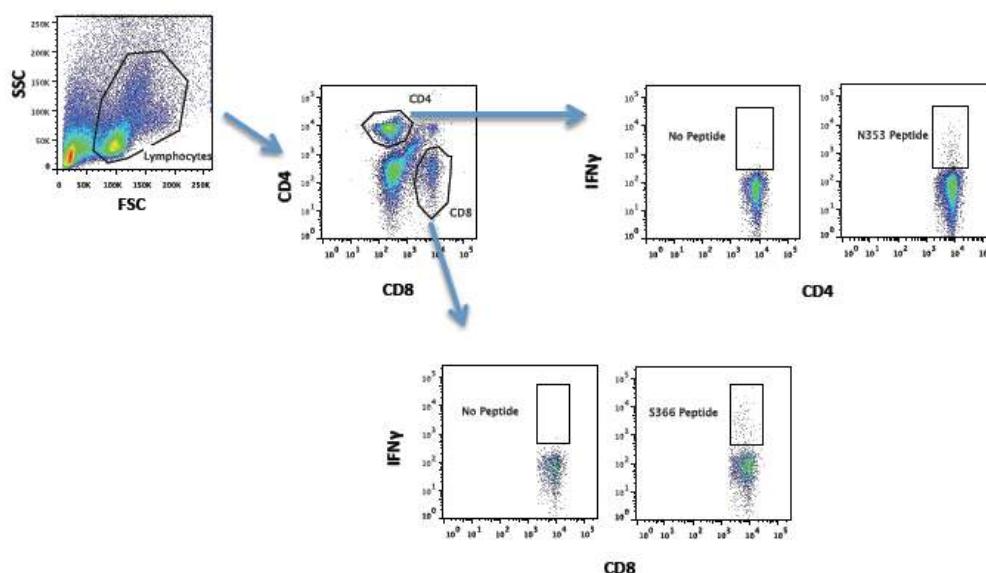


Figure 1. Strategy for identifying SARS-CoV specific T cell responses. Mice were infected with mouse-adapted SARS-CoV. After 6-8 days, mice were euthanized, lungs removed and single cell suspensions prepared for flow cytometry. Left hand panel shows mononuclear cell gate. CD4 and CD8 T cells were identified and assessed for IFN- γ expression after stimulation with peptides.

Recipes

1. Ketamine solution (10 ml)
1.75 ml ketamine
0.25 ml xylazine
8 ml 0.9% sodium chloride irrigation
Stored at room temperature
2. Digestion buffer (100 ml)

- 100 mg collagenase D
10 mg DNase I
2 ml FBS
1 ml glutamine
2.5 ml Hepes
5 ml penicillin streptomycin
89.5 ml HBSS
Stored at -20°C
3. RP10 (500 ml)
50 ml FBS
500 µl 2-Mercaptoethanol (from 50 mM stock made in H₂O)
450 ml RPMI medium 1640
Stored at 4°C
4. FACS buffer (500 ml)
1.7 ml sodium azide (from 30% stock made in H₂O)
20 ml FBS
480 ml PBS
Stored at 4°C
5. Cell surface staining mixture (100 µl = 1 sample)
1 µl rat serum
0.25 µl CD4 PerCP-Cyanine5.5
0.5 µl CD8a FITC
100 µl FACS buffer
6. Intracellular staining mixture (100 µl = 1 sample)
0.3 µl IFN γ APC
100 µl BD Perm/WashTM buffer

Acknowledgments

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Simultaneous Intranasal/Intravascular Antibody Labeling of CD4⁺ T Cells in Mouse Lungs

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[Abstract] CD4⁺ T cell responses have been shown to be protective in many respiratory virus infections. In the respiratory tract, CD4⁺ T cells include cells in the airway and parenchyma and cells adhering to the pulmonary vasculature. Here we discuss in detail the methods that are useful for characterizing CD4⁺ T cells in different anatomic locations in mouse lungs.

Keywords: Memory CD4⁺ T cell, Lung, Bronchoalveolar lavage fluid, Intranasal/intravascular antibody labeling, Flow cytometry

[Background] To distinguish memory T cells in the circulation and tissues, a method for intravascular staining of T cells has been developed (Anderson *et al.*, 2012). This method has been widely used to define memory T cells in many organs and tissues, including lungs, spleens and intestines. However, memory T cells in the respiratory tract are located in three unique anatomic locations, *i.e.*, airway, parenchyma and pulmonary vasculature. Intravascular staining cannot distinguish cells in the airway and parenchyma, since they are both isolated from the circulation and intravascularly-administered antibodies will not stain these two populations. We designed a simultaneous intranasal/intravascular antibody labeling assay that can label and distinguish cells in all three locations using minimal amount of antibodies.

Materials and Reagents

1. 1.5 ml microtubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 69715)
2. 1 ml insulin syringe fitted with 28 G × ½ needle (BD, catalog number: 329420)
3. Precision glide needles (20 G × 1) (BD, catalog number: 305175)
4. 5 ml polystyrene round bottom tubes (Corning, Falcon®, catalog number: 352054)
5. Precision glide needles (25 G × 5/8) (BD, catalog number: 305122)
6. 200 µl tips (Thermo Fisher Scientific, Invitrogen™, catalog number: AM12655)
7. 15 ml screw cap conical tubes (SARSTEDT, catalog number: 62.554.002)
8. 10 ml syringes (BD, catalog number: 309604)

9. 12 well cell culture plates (Corning, Costar®, catalog number: 3513)
10. 3 ml syringes (BD, catalog number: 309657)
11. 50 ml screw cap conical tubes (SARSTEDT, catalog number: 62.547.004)
12. Cell strainers (70 µm nylon) (Corning, Falcon®, catalog number: 352350)
13. 60 × 15 mm tissue culture dish (Corning, Costar®, catalog number: 353802)
14. 10 ml stripettes (Corning, Costar®, catalog number: 3548)
15. Gauze sponges (4 × 4 inch) (Pro Advantage by NDC, catalog number: P157118)
16. 0.2 µm filter (EMD Millipore, catalog numbers: SCGPU11RE and SLGP033RS)
17. 2.5 ml graduate transfer pipettes (RPI, catalog number: 147501-1S)
18. Absorbent pads (COVIDIEN, catalog number: 949)
19. Mouse
20. CD45-brilliant violet 510 (BV510) (Clone: 30-F11) (BioLegend, catalog number: 103138)
21. 1× Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14190-144)
22. CD90.2-APC-eFluor 780 (Clone: 53-2.1) (Affymetrix, eBioscience, catalog number: 47-0902)
23. Isoflurane (USP inhalation vapour, liquid) (Drugs, catalog number: 57319-559-06)
24. Ethanol (Sigma-Aldrich, catalog number: 459836)
25. Trypan blue solution (Thermo Fisher Scientific, Gibco™, catalog number: 15250061)
26. CD16/32-PerCP/Cy5.5 (Clone: 93) (BioLegend, catalog number: 101324)
27. CD4-FITC (Clone: RM4-5) (BioLegend, catalog number: 100510)
28. 2,2,2-tribromoethanol (Sigma-Aldrich, catalog number: T48402)
29. 2-methyl-2-butanol (Sigma-Aldrich, catalog number: 152463)
30. Nano-pure water (Thermo Fisher Scientific, Invitrogen™, catalog number: 10977015)
31. RPMI medium 1640 (Thermo Fisher Scientific, Gibco™, catalog number: 11875093)
32. HEPES (1 M) (Thermo Fisher Scientific, Gibco™, catalog number: 15630080)
33. L-glutamine (200 mM) 100× (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
34. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150)
35. MEM non-essential amino acids solution 100× (Thermo Fisher Scientific, Gibco™, catalog number: 11140050)
36. Sodium pyruvate 100× (Thermo Fisher Scientific, Gibco™, catalog number: 11360070)
37. Penicillin/streptomycin 100× (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
38. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
39. Collagenase D (Roche Diagnostics, catalog number: 11088882001)
40. DNase I (Roche Diagnostics, catalog number: 10104159001)
41. Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific, Gibco™, catalog number: 14025)
42. Sodium azide, NaN₃ (AMRESCO, catalog number: 0639)
43. Potassium bicarbonate, KHCO₃ (Sigma-Aldrich, catalog number: 237205)
44. Ammonium chloride, NH₄Cl (Sigma-Aldrich, catalog number: A9434)

45. EDTA-Na₂ (Sigma-Aldrich, catalog number: E9884)
46. BD Cytofix fixation solution (BD, catalog number: 554655)
47. Avertin (see Recipes)
48. Complete RPMI 1640 medium (see Recipes)
49. Digestion buffer (see Recipes)
50. FACS buffer (see Recipes)
51. ACK lysis buffer (see Recipes)

Equipment

1. Desiccator (SP Scienceware - Bel-Art Products - H-B Instrument, model: Space Saver Vacuum Desiccators)
2. Fume hood (LABSCAPE)
3. Pipetman P10 (Eppendorf, model: Research plus)
4. Pipetman P200 (Eppendorf, model: Research plus)
5. Pipetman P1000 (Eppendorf, model: Research plus)
6. Pipet aid (Eppendorf, model: Eppendorf Easypet 3)
7. Heat lamp (Whitehead Industrial, catalog number: 30715)
8. Mouse restrainer (Braintree Scientific, catalog number: TV-150 STD)
9. Spray bottle with 70% ethanol (SKS Science Products, catalog number: 0185-11)
10. Surgical scissors (Sklar Surgical Instrument, catalog number: 47-1246)
11. Tweezers (Sklar Surgical Instrument, catalog number: 66-7644)
12. Polystyrene foam (Regular polystyrene box top)
13. Rocker (Labnet, catalog number: S0500)
14. Refrigerated tabletop centrifuge (Beckman Coulter, model: Allegra 6R)
15. Hemocytometer (Thermo Fisher Scientific, catalog number: 99503)
16. Flow cytometer (BD, model: FACSVerse)

Software

1. Flowjo software (Version 10.0.7)

Procedure

- A. Simultaneous intranasal/intravascular antibody labeling (see Videos 1 and 2)
 1. Dilute 0.25 µg CD45-BV510 antibody in 100 µl DPBS in a 1.5 ml microtube (for intranasal labelling). Dilute 0.5 µg CD90.2-APC-eFuor 780 antibody in 300 µl DPBS in another 1.5 ml microtube and load the antibody into a 1 ml insulin syringe with a 28 G × ½ needle (for intravenous labelling). Place the microtube and the syringe on ice until use. (see Note 1)

2. Put a plastic desiccator in a fume hood. Place some gauze in the bottom chamber followed by 5 ml of isoflurane. (see Note 2)
3. Place a mouse on the grate inside the desiccator (see Note 3).
4. Load a P200 micropipette with 100 μ l of CD45-BV510 antibody.
5. Immediately after the rate of respiration slows, pick up the mouse, hold it vertically and slowly deliver 100 μ l of CD45-BV510 antibody onto the mouse nostrils. Hold the mouse upright so that the liquid on the nostrils is completely aspirated into the lungs.
6. Place the mouse back in the cage. After 2 min, turn on the heat lamp over the cage and warm up the mouse for 1 min.
7. Remove the mouse from the cage, put it in a mouse restrainer and inject 0.5 μ g CD90.2-APC-eFuor 780 antibody in 300 μ l DPBS through the tail vein with the 1 ml insulin syringe prepared in point 1.
8. Release the mouse from the restrainer and immediately anesthetize the mouse by intraperitoneal injection of 300 μ l of avertin solution. Secure the mouse in the palm of the hand and hold it horizontally, then insert the needle at a shallow angle tangential to the mouse and slowly dispense avertin solution into the peritoneal cavity.



Video 1. Intranasal administration of antibodies



Video 2. Intravenous administration of antibodies

B. Harvest cells from airway (see Video 3)



Video 3. Harvest of bronchoalveolar fluid, to obtain airway resident cells

1. Prepare a 1 ml syringe with 20 G × 1 needle. Carefully pass about 1.25 inches 20 G tubing onto the needle. Sharpen the end of the tubing with scissors. Load the syringe with 1 ml complete RPMI 1640 medium and put it on ice until use (Figure 1).



Figure 1. Representative pictures of syringe preparation and surgery for BALF lavage

2. When the mouse is fully anesthetized, typically within 2 min, immobilize it on a piece of polystyrene foam with absorbent pad on top by pinning each limb with a 25 G × 5/8 needle.
 3. Use a spray bottle filled with 70% ethanol to wet the fur of the mouse.
 4. Cut open the skin covering the abdominal and thoracic cavities through to the lower jaw. Use scissors to remove the tissue from the neck and expose the trachea.
 5. Use tweezers to separate the trachea from the underlying tissue and place a 200 μ l tip under the trachea.
 6. Use a razor to cut through $\frac{1}{2}$ of the trachea. Insert the 1 ml syringe with 20 G × 1 needle into the incision. Lavage for 4 times with complete RPMI 1640 medium. Collect the bronchoalveolar lavage fluid (BALF) in a 15 ml conical tube on ice. Typically 3-3.5 ml of BALF is recovered (Figure 1).
 7. Spin down cells for 5 min at 400 $\times g$ at 4°C in a bucket tabletop centrifuge and resuspend the cells in 1 ml of ice cold FACS buffer (see Note 5).
- C. Sacrifice the mouse and harvest cells from lung (see Video 4)



Video 4. Perfusing and harvesting lungs for cellular analysis

1. After collection of BALF, cutting open the abdominal cavity to expose the underside of the diaphragm. Cut through the diaphragm with scissors and then remove the ribcage to fully expose the heart and lung.
2. Fill a 10 ml syringe with ice cold sterile DPBS and attach a 25 G × 5/8 needle. Insert the needle into the left ventricle of the heart and smoothly dispense 5 ml of DPBS into the heart. In the meantime, use tweezers to break the right atria to allow blood to drain from circulation. Remove the needle from the left ventricle and insert it into the right ventricle to directly perfuse the lung with the remaining 5 ml of DPBS. (see Note 6)
3. Cut the heart away from the lung and then remove the lung from the thoracic cavity after cutting the trachea and any remaining connective tissue.
4. Place the lung into the well of a 12 well tissue culture plate filled with 2.5 ml of DPBS on ice.
5. Rinse the lung with DPBS and transfer it into another well without DPBS. Mince the lungs into very fine pieces using scissors.
6. Transfer minced lung with a 2.5 ml transfer pipette to 5 ml of digestion buffer in a 15 ml conical tube.
7. Place tubes on a rocker and gently rotate at room temperature for 30 min in the dark.
8. Place a 70 µm cell strainer into a 60 × 15 mm tissue culture dish.
9. Transfer lung tissue in digestion buffer to the cell strainer using a 2.5 ml transfer pipette. Gently press and dissociate tissue through strainer with the flat end of a 3 ml syringe plunger. Process tissues until there is only connective tissue remaining on the strainer and rinse the strainer with complete RPMI 1640 medium. Transfer the resulting suspension to a 50 ml conical tube.
10. Spin down lung cells for 5 min at 400 × g at 4°C in a bucket tabletop centrifuge.
11. Pour off supernatant and resuspend the cells in 3 ml of ACK buffer for 1 min to lyse the remaining red blood cells. Neutralize the ACK buffer with 30 ml of ice cold DPBS.
12. Spin down the cells for 5 min at 400 × g at 4°C and resuspend the cells in 5 ml of ice cold FACS buffer.

D. Cell staining

1. Count the cells from BALF and lung using a hemocytometer in the presence of trypan blue.
2. Spin down lung cells for 5 min at $400 \times g$ at 4°C and resuspend the cells in FACS buffer at 1 million cells per 50 μl .
3. Spin down cells in the BALF for 5 min at $400 \times g$ at 4°C . Since much fewer cells are recovered from airway, resuspend $1\text{-}5 \times 10^6$ cells per 50 μl FACS buffer as needed.
4. Dilute 0.25 μg CD4-FITC and 0.1 μg CD16/32 antibodies in 50 μl FACS buffer (see Note 4).
5. Gently mix 50 μl cells and 50 μl antibodies together in a FACS tube.
6. Incubate the cells in the dark for 15 min at 4°C .
7. Wash the cells once with 2 ml of FACS buffer at $400 \times g$ for 5 min at 4°C .
8. Remove the supernatant and resuspend the cells in 100 μl FACS buffer.
9. Pass the cells through a 70 μm cell strainer into new FACS tubes using a pipetman. Acquire FACS data using a flow cytometer and analyze data using Flowjo software (Figure 2). (see Note 7)
10. Flow cytometry gating strategy

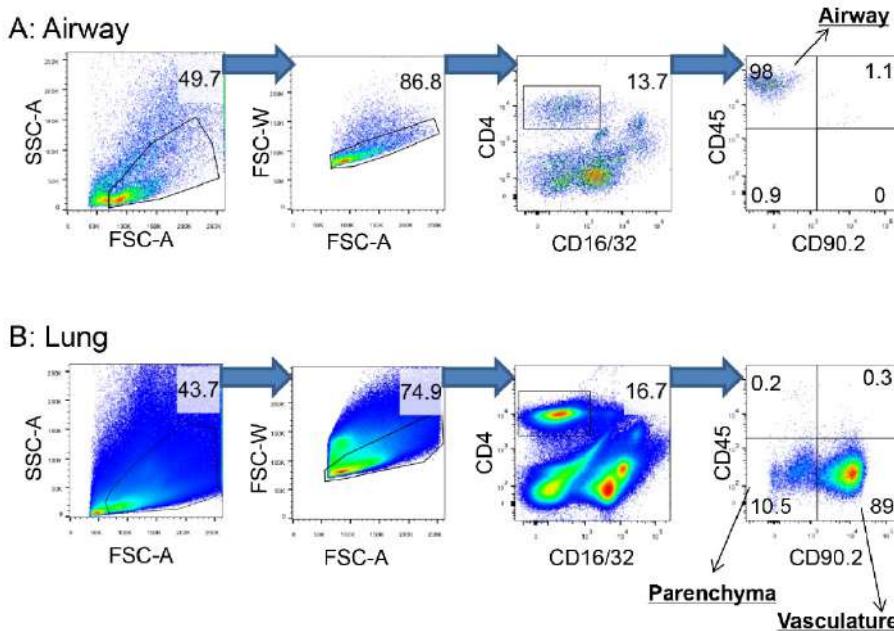


Figure 2. Gating strategy to distinguish CD4⁺ T cells in the airway, parenchyma and pulmonary vasculature. To localize memory CD4⁺ T cells in the respiratory tract, 0.25 μg of fluorochrome-conjugated CD45 and 0.5 μg of fluorochrome-conjugated CD90.2 antibody were injected into a SARS-CoV nucleocapsid protein-experienced mouse by i.n. and i.v. routes, respectively. Cells in the airway and lung were then harvested, stained and collected as described in Experimental Procedures and analysis using Flowjo software. A. CD4⁺ T cells in the airway: CD4⁺CD45⁺CD90.2⁻. B. CD4⁺ T cells in the parenchyma: CD4⁺CD45⁻CD90.2⁻. C. CD4⁺ T cells in the vasculature: CD4⁺CD45⁻CD90.2⁺. Data are representative of 10 independent experiments. (Zhao et al., 2016)

Notes

1. CD45 and CD90 are expressed by all CD4⁺ T cells. Both CD45 and CD90 antibodies can be used to stain CD4⁺ T cells in the lung and circulation. Phycoerythrin (PE), APC, BV (or their derivatives)-conjugated antibodies are preferentially chosen since these molecules have large molecular weights that will minimize airway/vascular leakage during *in vivo* labeling.
2. Make sure to tape over the center hole in the plastic desiccator grate because small mice are sometimes able to squeeze through this hole and jump into the bottom, isoflurane-filled chamber.
3. Here we used an antigen-experienced mouse that had been immunized with a construct expressing the SARS-CoV nucleocapsid protein (Zhao *et al.*, 2016). This protocol can be applied to study memory and effector as well as naïve CD4⁺ T cells in mouse lungs.
4. Cells can also be stained with other phenotypic (surface) antibodies or cultured for intracellular cytokine staining as previously described (Fett *et al.*, 2014).
5. For phenotypic staining, cells will be resuspended in FACS buffer. For intracellular staining, cell will be resuspended in complete RPMI 1640 medium.
6. The exterior surface of the lung will turn white after a good perfusion. Generally 5 ml PBS is sufficient.
7. This step is necessary to prevent clots formation in the flow cytometer. Other flow cytometers, such as LSR, Fortessa... can also be used to acquire cells.

Recipes

1. Avertin (250 ml)
5 g 2,2,2-tribromoethanol
5 ml 2-methyl-2-butanol
225 ml hot nano-pure water (95°C)
Stir for 2 h in a fume hood and adjust volume to 250 ml
0.2 µm filter, aliquot and store at -20°C
2. Complete RPMI 1640 medium (500 ml)
417 ml RPMI 1640 medium
12.5 ml 1 M HEPES
5 ml 100× L-glutamine
50 ml FBS
5 ml 100× MEM non-essential amino acids
5 ml 100× sodium pyruvate
5 ml 100× penicillin/streptomycin
0.5 ml 2-mercaptoethanol (50 mM in nano-pure H₂O, 0.2 µm filtered)
Store at 4°C
3. Digestion buffer (100 ml)

- 100 mg collagenase D
10 mg DNase I
2 ml FBS
1 ml 100× glutamine
2.5 ml HEPES (1 M)
1 ml 100x penicillin/streptomycin
Adjust volume to 100 ml with HBSS
Aliquot and store at -20°C
4. FACS buffer (500 ml)
- 1.7 ml sodium azide (from 30% stock made in nano-pure H₂O)
15 ml FBS
483.3 ml DPBS
Store at 4°C
5. ACK lysis buffer
- 1 g KHCO₃
8.3 g NH₄Cl
37.2 mg EDTA-Na₂
Adjust volume to 1,000 ml with nano-pure H₂O and filter through a 0.2 μm filter
Store at room temperature

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Virus Infection and Titration of SARS-CoV in Mouse Lung

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[Abstract] Two critical steps when investigating an animal model of a virus infection are consistently successfully infecting animals and accurately determining viral titers in tissue throughout the course of infection. Here we discuss in detail how to infect mice with SARS-CoV and then quantify the titer of virus in the lung.

Materials and Reagents

1. Mice (NCI) (Balb/c or C57BL/6, 4 weeks to 18+ months)
2. Vero E6 cells (ATCC, catalog number: CRL-1586)
3. Isofluorane (USP inhalation vapour; liquid) (NDC: 57319-559-06)
4. Dulbecco's modified eagle medium high glucose (DMEM) (Life Technologies, Gibco®, catalog number: 11965092)
5. 100 mg/ml ketaset III ketamine HCl injection (USP) (DEA Schedule II Drug) (NDC: 0856-2013-01)
6. 100 mg/ml AnaSed injection xylazine (Lloyd Laboratories, NADA number: 139-236)
7. 0.9% Sodium chloride irrigation (USP) (Baxter, catalog number: 2F7124)
8. 1x Dulbecco's phosphate buffered saline (DPBS) (Life Technologies, Gibco®, catalog number: 14190-144)
9. Formaldehyde solution (Sigma-Aldrich, catalog number: 252549) (37 wt.% in H₂O)
10. Crystal violet (Sigma-Aldrich, catalog number: C0775)
11. 25 mM DMEM with hepes (Life Technologies, Gibco®, catalog number: 32430)
12. 200 mM L-Glutamine (Life Technologies, Gibco®, catalog number: 25030-081)
13. FBS (Atlanta Biologicals, catalog number: S11150)
14. MEM non-essential amino acids (Life Technologies, Gibco®, catalog number: 11140-050)
15. 50 mg/ml gentamicin sulfate (Lonza, catalog number: 17-518Z)
16. Penicillin streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)
17. DMEM (Life Technologies, Gibco®, catalog number: 12100-046)
18. Agarose (optimized grade) (Research Products International, catalog number: A20090)
19. Ketamine solution (see Recipes)
20. Culture media (see Recipes)
21. Overlay media (see Recipes)

Equipment

1. Biosafety hood in a biosafety level 3 facility
2. Absorbent pads (Covidien, catalog number: 949)
3. 2 ml micro tube (Sarstedt AG, catalog number: 72.694.006)
4. 5 ml syringe (BD, catalog number: 309646)
5. 75 cm² cell culture flask (canted neck, 0.2 µm vent cap) (Corning, catalog number: 430641)
6. 12 well cell culture cluster (flat bottom with lid) (Corning, Costar®, catalog number: 3513)
7. 48 well cell culture cluster (flat bottom with lid) (Corning, Costar®, catalog number: 3548)
8. 10 ml stripette (Corning, Costar®, catalog number: 3548)
9. 35 ml closed tissue grinder system (Fisher Scientific, catalog number: 02-542-08)
10. Dessicator (Narang Medical, catalog number: P37.1517P)
11. Gauze Sponges (4 × 4 inch) (Pro Advantage® by NDC, catalog number: P157118)
12. 1 ml syringe (BD, catalog number: 309659)
13. Precision glide needle (25 G × 5/8) (BD, catalog number: 305122)
14. Spray bottle with 70% ethanol
15. Surgical scissors
16. Polystyrene foam
17. Pipetman P200 micropipette
18. Pipetman P1000 micropipette
19. CO₂ incubator
20. Pipet aid
21. Small metal weighing spatula
22. Tweezers
23. -80°C freezer

Procedure

A. Infection

1. Arrange materials in a ventilated hood. When using SARS-CoV, all procedures are performed in a biosafety hood in a BSL3 laboratory. When using isofluorane, always anesthetize animals in a fume or biosafety hood.
2. Thaw virus on ice then dilute in DMEM to the appropriate concentration. Each mouse receives 50 µl of virus.
3. Place several ply of gauze in the bottom of a plastic dessicator followed by 10 ml of isofluorane.
Note: Make sure to tape over the center hole in the plastic dessicator grate because small mice are sometimes able to squeeze through this hole and jump into the bottom, isofluorane filled chamber.

4. Load a P200 micropipette with 50 μ l of virus and then secure it in a safe position while placing a mouse on the grate inside the dessicator.
5. Immediately after the rate of respiration slows, pick up the mouse, hold it vertically and then slowly deliver 50 μ l of virus to both nostrils of the mouse. When mice are lightly anesthetized breathing will be deep enough that liquid on the nostrils should be completely aspirated into the lungs.

Note: The orifice size of the pipetman tips used matters significantly; tips with too small an orifice make it difficult to smoothly dispense virus onto the nostrils.

B. Removal and processing of lung tissue

6. A mouse is euthanized by intraperitoneal injection of 300 μ l ketamine/xylazine solution. To inject fluid into the intraperitoneal cavity, secure a mouse in the palm of the hand and hold it horizontally. Insert a 25 G \times 5/8 g needle at a shallow angle tangential to the mouse and slowly dispense ketamine/xylazine into the peritoneal cavity.
7. Once the mouse is fully anesthetized, place it on a square of absorbant padding on polystyrene foam and immobilize the mouse by pinning each limb with a 25 G \times 5/8 gauge needle.
8. Use a spray bottle filled with 70% ethanol to sterilize the fur of the mouse.
9. Use scissors to cut open the abdominal and thoracic cavities by first making an incision from the lower abdominal to the throat of the animal. Opening the abdominal cavity exposes the underside of the diaphragm. Use scissors to pierce through the diaphragm. Cut away the ribcage of the mouse and fully expose the heart and lungs. Cut the heart away from the lungs, and then remove the lungs by cutting the trachea and any remaining connective tissue.
10. After the lungs are removed from the thoracic cavity, use a 5 ml syringe filled with PBS to wash the blood from the outside of the lungs.
11. Place the lungs in a tissue grinder and grind them coarsely. Add 3 ml of PBS to the grinder and continue grinding the tissue to a homogenate.
12. Aliquot 1 ml of homogenate into 2 ml micro tubes, place on dry ice and then transfer to a -80°C freezer.

C. Virus titration

13. Vero E6 cells are split every 2 or 3 days at 1:4 or 1:6 dilutions respectively. Cells are maintained in a CO₂ incubator at 5% CO₂ and 37°C. Plan to have confluent T75 flasks ready the afternoon before tittering experiments. Split cells at a 1:4 dilution and then add media containing 200,000 cells to each well of a 12 well plate.
14. The next day, make dilutions of the virus in DMEM filled wells of a 48 well plate. Dilute 100 μ l of sample in 900 μ l of DMEM and so on until the sample is appropriately diluted. Plan to infect duplicate wells for each virus, at each dilution. When initially characterizing a SARS-CoV strain try testing the range of 1:10 dilutions from undiluted to 10⁷. Negative control wells are not required.

15. Prepare overlay medium as described below.
16. Remove media from the Vero E6 cells and then add 125 μ l of each sample dilution to be tested. This must be done rapidly so that the agarose mixture does not solidify before addition to the wells.
17. Return plates to the CO₂ incubator and then gently rock the plate back and forth several times every ten min for 30 min. Observe that rocking is sufficient so that the monolayer remains moist.
18. Remove the inoculum and add 500 μ l of overlay medium per well. When the agarose solidifies add 500 μ l of 2% serum culture media per well and return plate to the incubator.
19. On day three post-infection, remove plates from the incubator and add 1 ml of 25% formaldehyde diluted in PBS to each well. Fix cells for 20 min at room temperature and then suction off liquid using a pipet and pipet-aid. Remove agarose layer using a small spatula, being careful not to accidentally disrupt the monolayer.

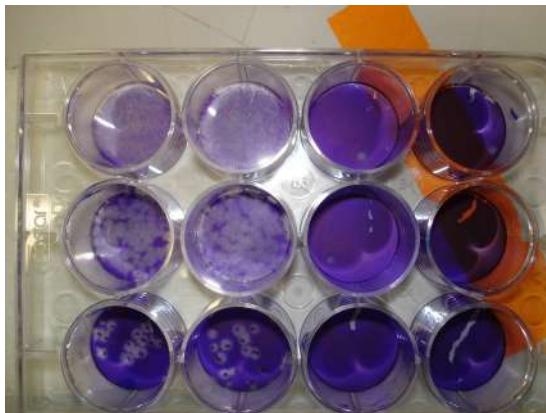


Figure 1. Visualization of plaques Monolayers of indicator cells and dilutions of virus were prepared. Samples were inoculated onto cells and plaques visualized as described above. Wells on right hand side of figure contain no plaques while the upper two rows in the first 2 columns illustrate nearly complete destruction of the monolayer. Clearly delineated plaques are shown in the bottom row.

20. Stain cells with 200 μ l of 0.1% crystal violet per well for at least 5 min, remove dye, wash once with ddH₂O, and count plaques.

D. Quantification of lung viral titer

21. Viral titers are expressed as Log₁₀ PFU/gram of tissue, calculated using the following equation:
$$\text{Log} [(\text{number of plaques} \times 10^{\text{dilution factor}} \times 1/\text{volume used to overlay cells} \times 3)/\text{weight of lungs}]$$
3 is the volume of PBS used to homogenize the tissue. For example let's hypothesize that there were 5 plaques in a well that had received a sample that was diluted by a factor of 1000. We add 0.125 L of sample to each well and assume a mean weight of 0.14 g for each mouse lung. The titer is then calculated as follows: $\text{Log} [(5 \times 10^3 \times 1 / 0.125 \times 3) / 0.14] = 5.9 \text{ Log}_{10} \text{PFU/gm}$

Recipes

1. Ketamine solution (10 ml)
1.75 ml ketamine
0.25 ml xylazine
8 ml 0.9% sodium chloride irrigation
Stored at room temperature in a double locked cabinet
2. Culture media (500 ml)
444.5 ml DMEM with HEPES (25 mM)
20 ml L-Glutamine
20 ml FBS
10 ml MEM non-essential amino acids
0.5 ml gentamicin sulfate
5 ml penicillin streptomycin
Stored at 4°C
3. Overlay medium
Note: Mix equal volumes of a and b immediately before placing on cells.
 - a. 2× medium
2× DMEM (made from powdered DMEM according to the instructions with 500 ml of H₂O instead of 1 L)
20 ml L-Glutamine
20 ml FBS
1 ml gentamicin sulfate
10 ml MEM non-essential amino acids
5 ml penicillin streptomycin
Incubate at 37°C on the day of the assay
 - b. Agarose
1.2% agarose, optimized grade in sterile ddH₂O. Agarose is autoclaved and stored at room temperature. On the day of use, agarose is melted using a microwave and placed at 56°C. It is mixed with 2× media and directly added to wells.

Acknowledgments

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Primer ID Next-Generation Sequencing for the Analysis of a Broad Spectrum Antiviral Induced Transition Mutations and Errors Rates in a Coronavirus Genome

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[Abstract] Next generations sequencing (NGS) has become an important tool in biomedical research. The Primer ID approach combined with the MiSeq platform overcomes the limitation of PCR errors and reveals the true sampling depth of population sequencing, making it an ideal tool to study mutagenic effects of potential broad-spectrum antivirals on RNA viruses. In this report we describe a protocol using Primer ID sequencing to study the mutations induced by antivirals in a coronavirus genome from an *in vitro* cell culture model and an *in vivo* mouse model. Viral RNA or total lung tissue RNA is tagged with Primer ID-containing cDNA primers during the initial reverse transcription step, followed by two rounds of PCR to amplify viral sequences and incorporate sequencing adaptors. Purified and pooled libraries are sequenced using the MiSeq platform. Sequencing data are processed using the template consensus sequence (TCS) web-app. The Primer ID approach provides an accurate sequencing protocol to measure mutation error rates in viral RNA genomes and host mRNA. Sequencing results suggested that β-D-N4-hydroxycytidine (NHC) greatly increased the transition substitution rate but not the transversion substitution rate in the viral RNA genomes, and cytosine (C) to uridine (U) was found as the most frequently seen mutation.

Keywords: Coronavirus, Antivirals, Next generation sequencing, Primer ID, Mutation

[Background] Next generation sequencing (NGS) has been extensively used in biomedical research for the last decade. When applying NGS to study intra-host viral populations for RNA viruses, modifications in library prep and sequencing protocols need to be considered. The virus titers (or viral loads) vary greatly from specimen to specimen. While conventional NGS platforms require 1-500 ng of DNA (or RNA) in a sequencing run, in most cases a clinical sample will have less than 100 fg of viral RNA, requiring that the viral RNA first be converted to cDNA using reverse transcriptase, followed by one or two rounds of PCR amplification to generate enough material for sequencing. However, the extensive cycles of PCR amplification will cause nucleotide mis-incorporation, recombination and amplification bias. Furthermore, the NGS platforms have relatively high error rates adding additional

uncertainty to the resulting sequences (Liu *et al.*, 2012).

We have developed the Primer ID NGS approach to overcome the errors and bias from the conventional NGS approaches (Jabara *et al.*, 2011; Zhou *et al.*, 2015). During the initial cDNA synthesis step, we use cDNA primers with an embedded 11-base degenerate block of nucleotides (the Primer ID) to tag each viral RNA template with a unique ID, a version of the approach of adding a unique molecular identifier (UMI). The Primer ID is carried on throughout the downstream amplifications and sequencing. After sequencing, all raw sequences with the same ID are collapsed to make a template consensus sequence (TCS). Each TCS is linked to an original template that was queried in the initial cDNA synthesis step, and the number of TCS shows the sequencing sampling depth of the viral population. By the construction of consensus sequence from multiple raw sequencing reads for each viral template, we can greatly reduce the PCR and sequencing errors. We have shown that the Primer ID approach can greatly reduce the sequencing error to 1 in 10,000 nucleotides (100-fold reduction from the raw sequencing reads) and also reveal the true sampling depth of the viral population, making it possible to accurately measure the substitution rates and diversity in the viral genomes in a population (Figure 1) (Zhou *et al.*, 2015). We have further developed a multiplexing Primer ID approach, allowing the sequencing of multiple regions of the viral genome in a single cDNA synthesis/PCR (Figure 2) (Dennis *et al.*, 2018).

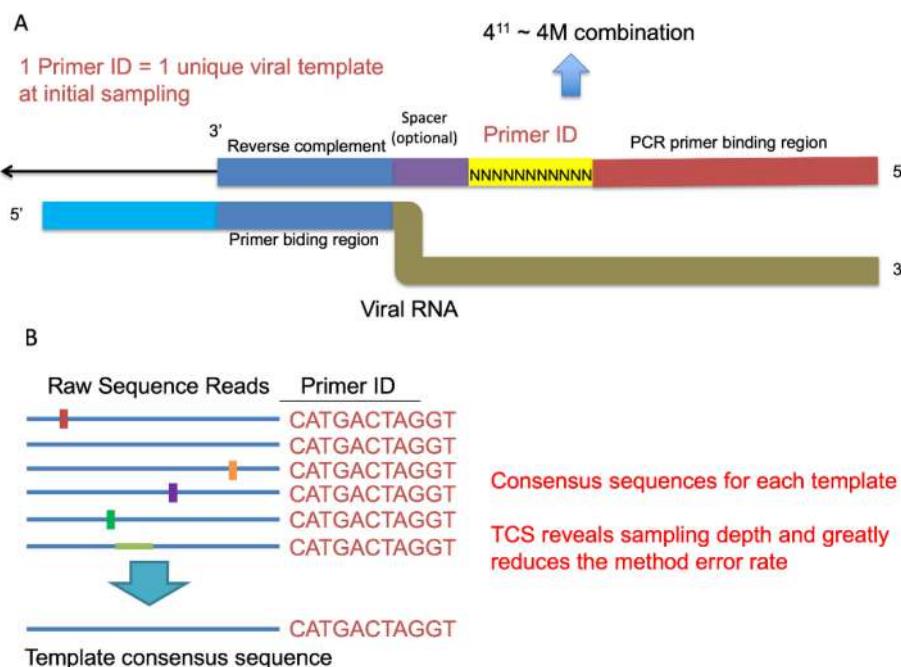


Figure 1. Primer ID Approach. A. The structure of the Primer ID primer and its binding to the viral RNA template. B. An example of creating the template consensus sequence from raw sequence reads.

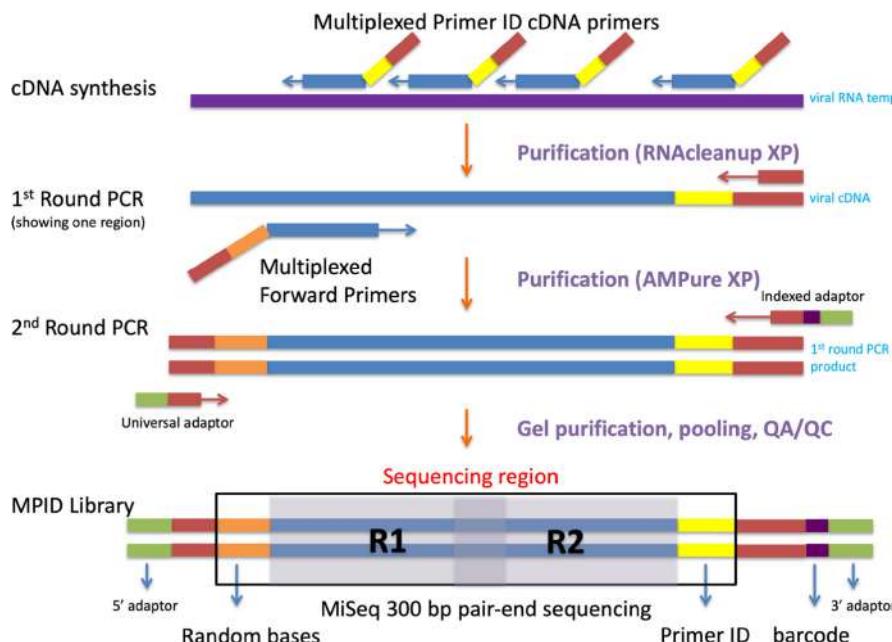


Figure 2. Library prep steps for the Multiplexed Primer ID sequencing. Biological sequence region in blue color, Primer ID region in yellow color, forward spacer region in orange, MiSeq barcode sequence region in purple.

This approach is ideal for studying intra-host RNA virus populations due to its ability to accurately characterize individual viral genomes. We have used this approach to determine the genetic structures of intra-host viral populations in HIV (Zhou *et al.*, 2016; Lee *et al.*, 2017; Dennis *et al.*, 2018; Abrahams *et al.*, 2019; Adewumi *et al.*, 2020; Council *et al.*, 2020), to study drug resistance mutations in HIV and HCV (Clutter *et al.*, 2017; Zhou *et al.*, 2018; Keys *et al.*, 2015), and to detect nucleotide mutation rates in single-stranded DNA molecule after heating (Lewis *et al.*, 2016).

In this report we describe a protocol using the Primer ID MiSeq sequencing to detect the mutagenic effect of EIDD-1931(β -D-N4-hydroxycytidine, NHC) and EIDD-2801 (an orally bioavailable prodrug of EIDD-1931 and is also recognized as MK-4482 or Molnupiravir) in the Middle East respiratory syndrome coronavirus (MERS-CoV) genome from *in vitro* cell culture and *in vivo* mouse models. In the cell culture sequencing experiment, we used a multiplexed Primer ID protocol to sequence multiple regions in the MERS-CoV ORF1b in a single library prep and sequencing reaction, while in the sequencing of total lung RNA from MERS-CoV infected mice, we used a multiplexed Primer ID protocol targeting both MERS-CoV genomes and two selected mouse mRNAs in a single library prep and sequencing reaction, in which we can directly compared the mutagenic effect induced by EIDD-2801 on MERS-CoV genomes and host mRNA. This protocol is not restricted to MERS-CoV and EIDD-1931 study only, but it can be used to detect viral RNA mutation rate in general.

Materials and Reagents

1. Pipette tips
2. Collection tubes (QIAGEN, catalog number: 19201, stored at room temperature)
3. Qubit Assay Tubes (ThermoFisher Scientific, Invitrogen, catalog number: Q32856, stored at room temperature)
4. SuperScript III One-Step RT-PCR System (ThermoFisher Scientific, Invitrogen, catalog number: 12574026, stored at -20°C)
5. Primer ID primers (Integrated DNA Technologies) at 100 µM in Tris-HCl buffer stored at -20°C
6. SuperScript III Reverse Transcriptase (ThermoFisher Scientific, Invitrogen, catalog number: 18080085, stored at -20°C)
7. RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific, Invitrogen, catalog number: 10777019, stored at -20°C)
8. Ribonuclease H (ThermoFisher Scientific, Invitrogen, catalog number: 18021071, stored at -20°C)
9. KAPA2G Robust PCR kits (Hotstart PCR kits with dNTPs) (Roche, catalog number: KK5516, stored at -20°C)
10. KAPA2G HiFi HotStart (with dNTPs) (Roche, catalog number: KK2502, stored at -20°C)
11. RNAClean XP (Beckman Coulter, catalog number: A63987, stored at 4°C)
12. AMPure XP (Beckman Coulter, catalog number: A63881, stored at 4°C)
13. Ethanol 200 Proof (Decon Laboratories, catalog number: 2716)
14. DNase/RNase-free water
15. MinElute Gel Extraction kit (QIAGEN, catalog number: 28606, stored at 4°C for MinElute columns, room temperature for other reagents)
16. Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Invitrogen, catalog number: Q32853, stored at 4°C for standard 1 and standard 2, room temperature for other reagents)
17. Experion DNA 12K analysis kit (Bio-Rad, catalog number: 7007108, stored at room temperature for the chips, 4°C for other reagents)
18. MiSeq Reagent Kit v3 (600-cycle) (Illumina, catalog number: MS-102-3003, stored at -20°C for box 1 and 4°C for box 2)
19. Sodium hydroxide (Sigma-Aldrich, catalog number: 221465, stored at room temperature)
20. Tris hydrochloride (Sigma-Aldrich, catalog number: 10812846001, stored at room temperature)
21. PhiX Control V3 (Illumina, catalog number: FC-110-3001, stored at -20°C)
22. 10 mM Tris-HCl with 0.1% TWEEN 20 (Teknova, catalog number: T7724, stored at room temperature)

Equipment

1. Pipettes
2. Magnetic rack
3. Thermal cycler (ThermoFisher Scientific, Applied Biosystems, catalog number: 4384638)
4. Centrifuge (Eppendorf, model: 5424)
5. DynaMag-2 Magnet (ThermoFisher Scientific, Invitrogen, catalog number: 12321D)
6. Qubit 2.0 Fluorometer (ThermoFisher Scientific, Invitrogen, catalog number: Q32866)
7. Experion Electrophoresis Station (Bio-Rad, catalog number: 7007010)
8. MiSeq System (Illumina, catalog number: SY-410-1003)

Software

1. Primer-BLAST (NCBI, <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Ye et al., 2012)
2. bcl2fastq pipeline (v.2.20.0) (Illumina,
https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)
3. TCS web app (UNC, <https://tcs-dr-dept-tcs.cloudapps.unc.edu/>)
4. TCS pipeline (v.1.3.8) (UNC, <https://github.com/SwanstromLab/PID>)
5. RUBY package viral_seq (v.1.0.8) (UNC, https://github.com/ViralSeq/viral_seq)

Procedure

We first design the primers for the multiplexed Primer ID library prep. After extraction of viral RNA from the tissue culture supernatants or total RNA from lung tissues, we construct MiSeq sequencing libraries using the multiplexed Primer ID library prep protocol from each specimen. The multiplexed Primer ID protocol allows us to make a sequencing library of multiple regions of the viral RNA in one reaction. We pool up to 24 barcoded libraries (from 24 different specimens) for each sequencing run. We use Illumina MiSeq V3 kit (600 cycle) for the sequencing. We choose paired-end sequencing to have longer read length and better error correction from overlapping regions over single-end sequencing.

A. Primer ID cDNA primer and PCR primer design

1. Design primer sequences using NCBI Primer-BLAST.
 - a. Use ORF1b region of the reference genome MERS-CoV NC_019843 as “PCR template” in the Primer-BLAST interface to design primers for MERS-CoV. Use mouse interferon-induced protein with tetratricopeptide repeats 2 (Ifit2, reference: NM_008332), interferon-induced protein with tetratricopeptide repeats 3 (Ifit3, reference: NM_010501), ISG15 ubiquitin-like modifier (Isg15, reference: NM_015783), interferon-induced protein with tetratricopeptide repeats 1 (Ifit1, reference: NM_008331) and chemokine (C-X-C motif)

- ligand 10 (Cxcl10, reference: NM_021274) genomes as templates to design primers for mouse mRNA.
- b. Set PCR product size as Min: 400 bp and Max: 600 bp.
 - c. Set Primer melting temperatures as Min: 52, Opt: 55, Max: 58.
 - d. Select 3-4 pairs of primers for each template region.
2. Use SuperScript III one-step RT-PCR system to test Primers with best amplification efficiency for each region.
 - a. Use MERS-CoV viral RNA from tissue culture described above for MERS-CoV primer testing. Use extracted total mouse lung RNA as template for mouse mRNA primer testing. Make serial 1:10 titrations of the template RNA in DNase/RNase-free water as RT-PCR templates. Make dilutions up to 10^{-6} .
 - b. Use following recipe to make one one-step RT-PCR mixture (total volume of 10 μ l) in PCR tubes.
 - 5 μ l of 2 \times buffer
 - 1.8 μ l of DNase/RNase-free water
 - 0.4 μ l of Forward primer (20 μ M)
 - 0.4 μ l of Reverse R (20 μ M)
 - 0.4 μ l of Enzyme mix
 - 2 μ l of Template RNA
 - c. Use the following thermal cycler condition for RT-PCR.
 - i. 50°C for 30 min
 - ii. 94°C for 2 min
 - iii. 40 cycles of:
 - 94°C for 15 s
 - 55°C for 30 s
 - 68°C for 30 s
 - iv. 68°C for 5 min
 - d. Examine the PCR products using gel electrophoresis with 1% agarose gel.
 - e. Figure 3 shows the example gel image of amplicons from tested primers for MERS-CoV.

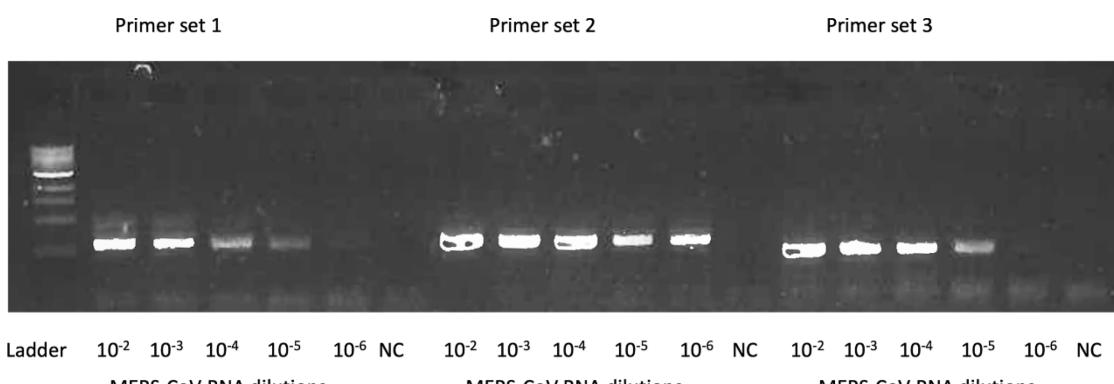


Figure 3. An example one-step RT-PCR gel image for the primer design. Three sets of

primers targeting same MERS-CoV genome were tested. A serial dilution of MERS-CoV RNA up to 10^{-6} dilution was used to test the primers. The SSIII one-step PCR system was used for the amplification. PCR products were examined by gel electrophoresis with 1% agarose gel. Primer set 2 had the best amplification efficiency thus it was selected for downstream Primer ID primer design. NC, no-reaction control.

3. Design the Primer ID cDNA primers and other primers for the MiSeq library prep.
 - a. Design cDNA primers by attaching the Primer ID (a degenerate block of 11-base long sequence) and adaptor sequence to the reverse primers.
 - b. Forward primer of the 1st round PCR is designed by joining a common sequence "GCCTCCCTCGGCCATCAGAGATGTGTATAAGAGACAGNNNN" on the 5' end with the forward primer sequencing designed at the step a) on the 3'. The Ns in the joined primer sequences are random sequences to increase the sequencing quality.
 - c. Reverse primer of the 1st round PCR is designed against the adaptor sequence section of the Primer ID cDNA primer, and is universal regardless of regions (Table 1).
 - d. The 2nd round PCR primers are designed to incorporate the MiSeq sequencing adaptors with barcodes.
 - e. Order primers designed above in Steps A1a-A1d from Integrated DNA Technologies (IDT). Request hand-mixing of the precursors for the degenerate block of sequence of the cDNA primers to have an even distribution of bases at each position.
 - f. Table 1 shows the complete list of primers used in this protocol including primers for Primer ID MiSeq library prep and customized MiSeq sequencing primer. Table 2 shows the sequences of the indexed primers.

Notes:

1. *Primer-BLAST is not necessary to design primers. Primers can be manually designed based on known reference genomes.*
2. *Hand-mixing of nucleotide precursors prior to Primer ID cDNA primer synthesis is important to ensure the degenerate sequences have an even distribution of bases (A, C, G, T) at each position of the degenerate block.*

Table 1. Primers used for MiSeq library prep and sequencing. Primer sequences with gene-specific regions are highlighted in blue. They can be replaced with other gene-specific primer sequences when designing primers for other viruses.

Primer	5'-3' sequence	Comment
41R_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT ATGACCTT CCTGTTGCTTCT	cDNA primer. Targeting 20331-20350 on the reference genome.
nsp10_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT CCTAAAGA CGACATCAGTGG	cDNA primer. Targeting 13488-13507 on the reference genome.
nsp12_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT ATAGCCAA AGACACAAACCG	cDNA primer. Targeting 15983-16002 on the reference genome.
nsp14_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT GAACATC GACAAAGAAAGGG	cDNA primer. Targeting 18715-18734 on the reference genome.
ifit3_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT TTCAGCC ACTCCTTATCCC	cDNA primer. Targeting mice IFIT3 mRNA.
isg15_PID11*	GTGACTGGAGTCAGACGTGTGCTCTCCGATCTNNNNNNNNNCAGT GGGCCTT TAGGCCATACTC	cDNA primer. Targeting mice ISG15 mRNA.
41F_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN GCTACAAGTTCGTCC TTTGG	1 st round PCR forward primer. Targeting 19812-19831 on the reference genome
nsp10_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN TGCTCAGGTGCTAAG CGAAT	1 st round PCR forward primer. Targeting 12983-13002 on the reference genome
nsp12_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN ATAGGCTTCGATGTT GAGGG	1 st round PCR forward primer. Targeting 15388-15407 on the reference genome
nsp14_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN ATTGCAAGCTGGTTC TAACA	1 st round PCR forward primer. Targeting 18260-18279 on the reference genome
ifit3_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN CGATCCACAGTGAAC AACAG	1 st round PCR forward primer. Targeting mice IFIT3 mRNA.
isg15_AD	GCCTCCCTCGGCCATCAGAGATGTATAAGAGACAGNNNN TGGGACCTAAAGGTG AAGATG	1 st round PCR forward primer. Targeting mice ISG15 mRNA.

Table 1. Continued

Adapter R	GTGACTGGAGTTCAGACGTGTGCTC	1 st round PCR reverse primer
Universal Adapter	AATGATAACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGA TGTG	2 nd round PCR forward primer with Illumina adapter sequence
Indexed Adapter**	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTG TGCTC	2 nd round PCR reverse primer with Illumina adapter sequence and indices (NNNNNNNN)
Old Nextera	GCCTCCCTCGGCCATCAGAGATGTGTATAAGAGACAG	Customized sequencing primer

* cDNA primer has a block of degenerate nucleotides (Ns) as the Primer ID.

** Ns in the Indexed Adapter primers are a set of 24 pre-designed barcodes for multiplexing samples for one MiSeq run. See the index sequences and adapter sequences in Table 2.

Table 2. Indexed adapter sequences. Note the index sequences are the reverse complement sequences of the highlighted (red) region in the adapter sequences

Indexed Adapter	Index	Index Sequence	Sequence (5'-3')
PCR Primer, Index 1	1	ATCACGA	CAAGCAGAAGACGGCATACGAGA TCGTGAT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 2	2	CGATGTA	CAAGCAGAAGACGGCATACGAGA TACATCG GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 3	3	TTAGGCA	CAAGCAGAAGACGGCATACGAGA TGCCTAA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 4	4	TGACCAA	CAAGCAGAAGACGGCATACGAGA TTGGTCA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 5	5	ACAGTGA	CAAGCAGAAGACGGCATACGAGA TCACTGT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 6	6	GCCAATA	CAAGCAGAAGACGGCATACGAGA TATTGGC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 7	7	CAGATCA	CAAGCAGAAGACGGCATACGAGA TGATCTG GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 8	8	ACTTGAA	CAAGCAGAAGACGGCATACGAGA TTCAAGT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 9	9	GATCAGA	CAAGCAGAAGACGGCATACGAGA TCTGATC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 10	10	TAGCTTA	CAAGCAGAAGACGGCATACGAGA TAAGCTA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 11	11	GGCTACA	CAAGCAGAAGACGGCATACGAGA TGTAGCC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 12	12	CTTGTAA	CAAGCAGAAGACGGCATACGAGA TTACAAG GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 13	13	TCCATAA	CAAGCAGAAGACGGCATACGAGA TTATGGA GTGACTGGAGTTCAGACGTGTGCTC

			C
PCR Primer, Index 14	14	GTACTAA	CAAGCAGAAGACGGCATACGAGA TTAGTAC GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 15	15	ACAGTAA	CAAGCAGAAGACGGCATACGAGA TTACTGT GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 16	16	CTCATGA	CAAGCAGAAGACGGCATACGAGA TCATGAG GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 17	17	ACGATAA	CAAGCAGAAGACGGCATACGAGA TTATCGT GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 18	18	TGCAGAA	CAAGCAGAAGACGGCATACGAGA TTCTGCA GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 19	19	TTCATAA	CAAGCAGAAGACGGCATACGAGA TTATGAA GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 20	20	TGCTGTA	CAAGCAGAAGACGGCATACGAGA TACAGCA GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 21	21	TATCACCA	CAAGCAGAAGACGGCATACGAGA TGTGATA GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 22	22	TGGATAA	CAAGCAGAAGACGGCATACGAGA TTATCCA GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 23	23	CGCATTAA	CAAGCAGAAGACGGCATACGAGA TAATGCG GTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 24	24	GCCTTAA	CAAGCAGAAGACGGCATACGAGA TTAAGGC GTGACTGGAGTTCAGACGTGTGCT C

B. Primer ID MiSeq library prep

1. cDNA synthesis
 - a. Use viral RNA extracted from cell culture supernatants from infected cells or total lung tissue RNA from infected mice as amplification templates. cDNA synthesis step should be performed in BSL2+ facility until after the Ribonuclease H step.
 - b. Completely thaw, vortex and spin down 5× buffer, dNTPs and DTT.
 - c. Make cDNA primer working mix from the 100 μM primer stock. The final concentration for each primer is 10 μM in the primer mix.
 - i. Determine regions for sequencing.
 - ii. Add 10 μl of each cDNA primer (no more than 5 different primers to multiplex) to an Eppendorf microcentrifuge tube, and add DNase/RNase-free water to a total volume of 100 μl.
 - iii. Increase the amount of primers and DNase/RNase-free water proportionally if larger volume of primer mix is needed.
 - d. Pipette the following components into a 0.5 ml RNase-free PCR tube.
2 μl of dNTP mix (10 mM each)
1 μl of Primer ID cDNA primer mix (10 μM each)
23 μl of RNA template (vRNA or total lung RNA)
 - e. Heat the PCR tube on a thermal cycler at 65°C for 5 min, then cool down to 4°C for 2 min with lid closed (lid temperature at 105°C).
 - f. Add the following components to the tube. If doing the cDNA synthesis in batch, make a master mix and add 14 μl of the mixture to each tube.
8 μl of 5× buffer
2 μl of DTT (100 mM)
2 μl of RNaseOUT (40 U/μl)
2 μl of SII reverse transcriptase (200 U/μl)
 - g. Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the cDNA synthesis.
55°C for 1 h
50°C for 1 h
70°C for 15 min
 - h. To each tube, add 0.5 μl (1U) Ribonuclease H, incubate at 37°C for 20 min. After this step, the cDNA can be handled on a regular workbench (BSL1).
 - i. Purify cDNA with RNAClean XP.
 - i. Resuspend the beads by vortexing and take an aliquot out. Keep in room temperature for at least 30 min before use.
 - ii. Resuspend the beads thoroughly by vortexing. Add 28 μl of RNAClean XP beads to 1.5 ml tubes. Transfer all of the cDNA reaction (40.5 μl) to each tubes with beads.
 - iii. Mix the RNAClean XP and sample thoroughly by pipette mixing 15 times. Vortexing is not recommended. Let the tube incubate at room temperature for 20 min. Spin down briefly if

- samples touches the upper part of the tube.
- iv. Place the tube onto the magnetic tube rack for 5 min to separate the beads from solution.
 - v. Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.
 - vi. Dispense 400 μ l of 70% ethanol into the tube and incubate for 30 s at room temperature. Aspirate out the ethanol and discard. Repeat for a total of three washes. It is important to perform these steps with the tube situated on the magnetic rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
 - vii. Let the reaction tube air-dry up to 10 min on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. However, over drying the sample may result in a lower recovery.
 - viii. Remove tube from magnetic rack and resuspend beads in 24 μ l DNase/RNase-free water by pipetting up and down. Place tube back on rack and leave for 3 min.
 - ix. Pipette the eluent (23.5 μ l) from the tube while it is situated on the magnetic tube rack.
2. First round PCR amplification with KAPA2G Robust PCR kit.
In the first round PCR, we amplify all the cDNA we make from the previous step using a mixture of forward primers targeting multiple regions of the genome.
 - a. Make forward primer working mix from the 100 μ M primer stock.
 - i. Determine regions of primer binding based on the initial choice of placement of the cDNA primers.
 - ii. Add 10 μ l of each forward primer (no more than 5 primers) to an Eppendorf microcentrifuge tube, and add DNase/RNase-free water to a total volume of 100 μ l.
 - iii. Increase the amount of primers and DNase/RNase-free water proportionally if larger volume of primer mix is needed.
 - b. Completely thaw, vortex and spin down reagents (except for enzyme) before use.
 - c. Use the following recipe to make one first round PCR mix (total volume of 50 μ l).
10 μ l of 5 \times Buffer A
10 μ l of Enhancer
1 μ l of dNTPs
2.5 μ l of Forward primer mix
2.5 μ l of adapter R primer
0.5 μ l of KAPA Robust polymerase
23.5 μ l of template cDNA
 - d. Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the first round PCR amplification.
 - i. 95°C for 1 min
 - ii. 25 cycles of:
95°C for 15 s

- 58°C for 60 s
72°C for 30 s
iii. 72°C for 3 min
- e. Purify PCR products using AMPure XP cleanup beads.
- Vortex the 1 ml aliquot of AMPure XP cleanup beads and remove the needed volume. Keep in room temperature for at least 30 min before use.
 - Transfer the first round of PCR reactions into 1.5 ml DNase-free tubes.
 - Resuspend the beads. Add 35 µl AMPure XP beads to each PCR reaction.
 - Mix the AMPure XP and sample thoroughly by vortexing. Let the tube incubate at room temperature for 5 min before proceeding to the next step (incubate off the rack).
 - Place the tube onto the magnetic tube rack for 5 min to separate the beads from solution.
 - Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.
 - Dispense 400 µl of 70% ethanol into the tube and incubate for 30 s at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes. It is important to perform these steps with the tube situated on the rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
 - Let the reaction tube air-dry up to 10 min on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery.
 - Remove tube from rack and resuspend beads in 50 µl DNase-free water by pipetting up and down. Place tube back on rack and leave for 3 min.
 - Pipette the 45 µl eluent from the tube while it is situated on the magnetic tube rack. Transfer the eluent to a new 1.5 ml microcentrifuge tube.
4. Second round of PCR amplification with KAPA2G HiFi PCR kits.
- Complete thaw, vortex and spin down reagents (except for enzyme) before use.
 - Use the following recipe to make one second round PCR mix (total volume of 25 µl).

10 µl of 5× KAPA HiFi Buffer
1 µl of dNTPs
1 µl of Universal Adapter
1 µl of indexed Adapter
0.5 µl of KAPA HiFi polymerase
2 µl of purified DNA from the first round PCR.
 - Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the cDNA synthesis.
 - 95°C for 2 min
 - 35 cycles of:
98°C for 20 s

- 63°C for 15 s
72°C for 30 s
iii. 72°C for 3 min
5. Gel purification of PCR products using QIAGEN MinElute Gel extraction kit.
 - a. Before gel extraction, run 2 µl PCR products on 1% agarose gel to check if the library has the right size.
 - b. Make 1.2% (w/v) agarose gel with ethidium bromide (final concentration of 0.2-0.5 µg/ml).
 - c. Load all the second round PCR products on the 1.2% agarose gel. Adjust the voltage based on the size of the gel. The ideal electric field strength is 4 V/cm. Run the gel for 30 min.
 - d. Excise target DNA fragments of the right size (500 bp to 1 kb) on a non-UV blue light transilluminator.
 - e. Weigh the gel; add 3 volumes of Buffer QG to 1 volume of gel.
 - f. Incubate at 50°C for 10 min to completely dissolve the gel fragment. Vortex every 2-3 min to help dissolve.
 - g. Check the color of the gel solution (should be yellow, otherwise add 10 µl 3 M sodium acetate of pH 5.0).
 - h. Apply the sample to the MinElute column and centrifuge at maximum speed for 1 min.
 - i. Transfer the column to a new collection tube, discard the filtrate, add 500 µl buffer QG and centrifuge at maximum speed for 1 min.
 - j. Transfer the column to a new collection tube, discard the filtrate, add 0.75 ml buffer PE, incubate for 5 min at room temperature, centrifuge at maximum speed for 1 min.
 - k. Transfer the column to a new collection tube, discard the filtrate, centrifuge at maximum speed for additional 3 min.
 - l. Put the column in a new 1.5 ml tube, add 10 µl buffer EB. Stand for 4 min, centrifuge at maximum speed for 2 min.
 6. Quantification, pooling and QA/QC.
 - a. Quantify each gel-purified library using Qubit dsDNA BR Assay kit, following the [manufacturer's protocol](#). Use 1 µl of the library for each quantification.
 - b. Pool the quantified individual libraries in equal amounts. We suggest pooling a total number of 20-24 libraries, each with 100 to 150 ng DNA per library. Use the maximum available volume of libraries if the calculated volume exceeds the actual volume.
 - c. Purify the pool of libraries with AMPure XP beads following the protocol above in the First Round PCR section. The volume of AMPure beads to use equals the volume of pooled libraries multiplied by 0.7.
 - d. Quantify the purified pooled libraries using Qubit dsDNA BR Assay kit, following the manufacturer's protocol.
 - e. Run the pooled libraries with the Experion Automated Electrophoresis System with DNA 12k analysis kit following the [manufacturer's protocol](#). An example of gel image of Experion is shown in Figure 4.
 - f. Check the size of DNA peaks on the Experion gel image. Make sure there is no visible peak

around 100 bp (primer dimer). If it is present, purify the pooled libraries with AMPure XP beads again and repeat the quantification and Experion electrophoresis.

- g. Calculate the average DNA size (bp) of the library pool based on the Experion molarity (nM) and Experion concentration (ng/ μ l) using the following equation. Do NOT use the Qubit concentration to calculate the average DNA size.

$$\text{avg.DNA size} = \frac{\text{Experion Concentration}}{\text{Experion Molarity} \times 650} \times 10^6$$

Notes:

1. It is important to set up the cDNA reaction in a separate room from where the PCR amplification takes place to avoid potential contamination from PCR products.
2. Viral RNA in this protocol that have very little quantity may not be measurable. qRT-PCR is usually recommended to confirm the copy numbers of viral RNA before library prep. We recommend at least 1,000 copies of input viral RNA for each library prep. However, this protocol may still work with less than 1,000 copies of input viral RNA, with compromised sampling depth.
3. Primer mix is only needed when multiplexed regions are sequenced. It is possible to use one set of cDNA/forward primers at 10 μ M concentration when sequencing only one region.
4. Multiplexed regions normally should not overlap as this may inhibit the efficiency of priming and/or amplification.
5. Include one negative control and possibly one positive control for each batch of library prep.
6. It is recommended to aliquot the RNAClean XP beads and the AMPure XP beads into 1 ml aliquots. It is very important to warm the beads in room temperature for 30 min before use.
7. The ratio of the volume of beads to cDNA/DNA is 0.7. It is optimized to remove long Primer ID cDNA primers or PCR primers.
8. We recommend a second round of cDNA purification for some clinical specimens, especially plasma samples, to remove inhibitors of the downstream amplification. After the cDNA is eluted in water (in this case, use 40 μ l water), add 28 μ l of new RNAClean XP beads and purify the cDNA for a second time.
9. Residual beads in the cDNA or PCR products eluent won't affect the downstream amplification.
10. First and second round PCR amplifications use two different KAPA PCR kits. The enzyme and buffer tubes look similar. It is very important to use the right buffer with the right enzyme for PCR 1 and PCR 2.
11. Save the first round PCR product in the 1.5 ml tube in -20 °C, in case it is needed to repeat PCR2.
12. Make sure to excise the fragment of the right size during gel extraction. Occasionally larger fragments (1.5-3 kb) can be present on a gel, which should not be extracted.
13. Library concentration normally should be at least 20 ng/ μ l. Discard libraries with concentration less than 10 ng/ μ l as they are less likely to have high quality sequences.
14. Qubit 2.0 fluorometer has been discontinued. Qubit 4.0 fluorometer is available at the time this protocol is written.

15. *Qubit dsDNA HS Assay kit is not recommended to quantify the libraries. The HS kits have an upper detection limit of 100 ng/ μ l. In some cases the library concentration can be higher than 100 ng/ μ l.*
16. *The Bio-Rad Experion system is used in this protocol but it is close to obsolete. A better substitution is Agilent TapeStation System (Agilent, catalog number: G2991AA). The TapeStation system can directly measure the average DNA size of the library pool.*

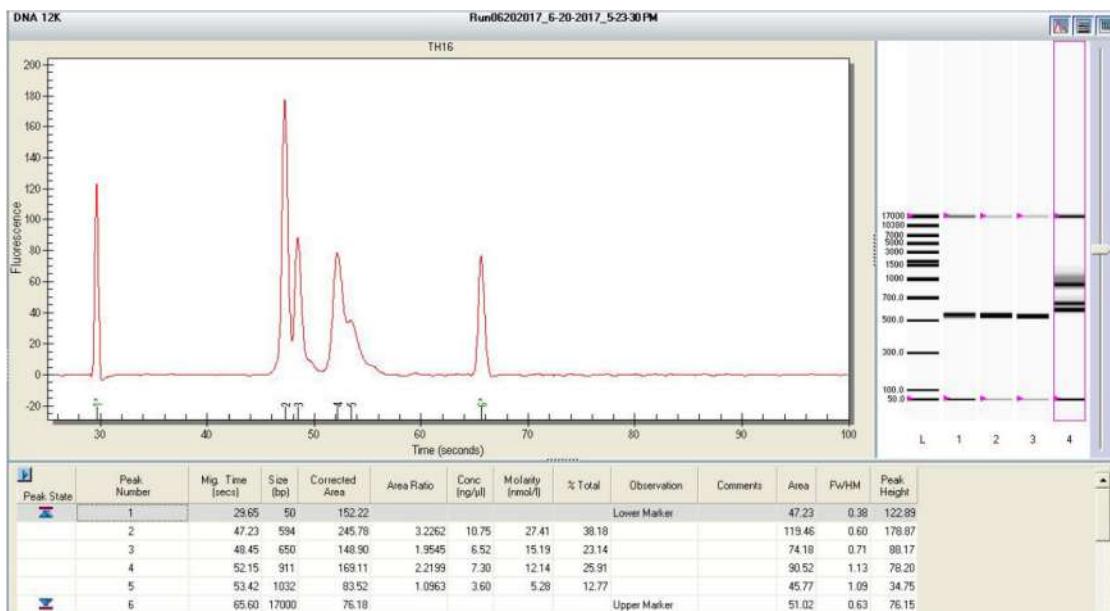
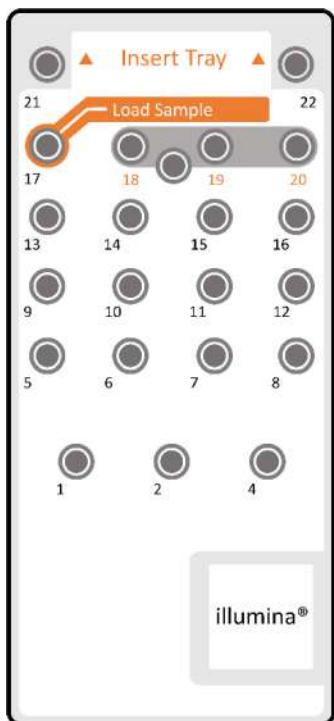


Figure 4. An example of Experion gel image

C. MiSeq sequencing run

The MiSeq is an integrated instrument that performs clonal amplification, DNA sequencing based on sequencing-by-synthesis, and data analysis with base calling, alignment, variant calling, and reporting in a single run. In our protocol, we use the MiSeq reagent kit V3 (600 cycle) for sequencing. Figure 5 shows the MiSeq reagent cartridge and content by well numbers. This step is modified based on the MiSeq V3 protocol.



Position	Reagent	Description
1	IMT	Incorporation Mix
2	USM	Scan Mix
4	CMS	Cleavage Mix
5	AMS1	Amplification Mix, Read 1
6	AMS2	Amplification Mix, Read 2
7	LPM	Linearization Premix
8	LDR	Formamide
9	LMX1	Linearization Mix
10	LMX2	Read 2 Linearization Mix
11	RMF	Resynthesis Mix
12	HP10	Read 1 Primer Mix
13	HP12	Index Primer Mix
14	HP11	Read 2 Primer Mix
15	PW1	Laboratory-grade water
16	PW1	Laboratory-grade water
17	Empty	Load Samples (Reserved for sample libraries)
18	Empty	Optional use for custom Read 1 primer
19	Empty	Optional use for custom Index Read primer
20	Empty	Optional use for custom Read 2 primer
21	PW1	Laboratory-grade water
22	Empty	Empty

Figure 5. MiSeq Reagent Cartridge and content by well numbers

1. Prepare the reagent cartridge.
 - a. Remove the reagent cartridge from -20°C storage.
 - b. Place the reagent cartridge in a water bath containing only enough room temperature, deionized water to submerge the base of the reagent cartridge. Do not allow the water to exceed the maximum water line printed on the reagent cartridge.
 - c. Allow the reagent cartridge to thaw in the room temperature water bath for approximately 60 min or until completely thawed.
 - d. Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge, if necessary.
 - e. Invert the reagent cartridge ten times to mix the thawed reagents, and visually inspect that all positions are thawed.
 - f. Visually inspect the reagent marked IMT (incorporation mix) to make sure that it is fully mixed and free of precipitates.
 - g. Gently tap the cartridge on the bench to dislodge water from the base of the cartridge and reduce air bubbles in the reagents.

Note: The MiSeq sipper tubes go to the bottom of each plate well to aspirate the reagents so it is important that there are no air bubbles stuck at the bottom of the reagent wells.

- h. Place the reagent cartridge on ice or set aside at 2°C to 8°C until you are ready to load the sample onto the reagent cartridge and set up the run.
2. Prepare the libraries.
 - a. Denature DNA
 - i. Prepare a fresh dilution of 0.2 N NaOH on the day of sample loading. This is essential in

- order to completely denature samples for cluster generation on the MiSeq. Always start with a concentrated stock of NaOH that is 10 N or higher, or you may get a decrease in cluster density.
- ii. Calculate the molarity of the pooled library based on the Qubit concentration and average DNA size, dilute the pooled library to 4 nM.
 - iii. Combine the following volumes of sample DNA and 0.2 N NaOH in a microcentrifuge tube:
 - 4 nM sample DNA (5 µl)
 - 0.2 N NaOH (5 µl)
 - iv. Vortex briefly to mix the sample solution, and then briefly centrifuge the sample solution.
 - v. Incubate for five minutes at room temperature to denature the DNA into single strands.
 - vi. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA to result in a 20 pM denatured library:
 - Denatured DNA (10 µl)
 - Pre-chilled HT1 (990 µl) (Shipped frozen in the MiSeq reagent cartridge box)
 - vii. Place the denatured DNA on ice until you are ready to proceed to final dilution.
- b. Dilute Denatured DNA
- i. Dilute the denatured DNA to 8 pM concentration using the following volumes:
 - 20 pM DNA (400 µl)
 - Pre-chilled HT1 (600 µl)
 - ii. Vortex briefly to mix the DNA solution.
 - iii. Pulse centrifuge the DNA solution.
 - iv. Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge directly or after adding the PhiX control.
- c. Denature and Dilute PhiX control
- i. PhiX is used as an internal sequencing control provided by Illumina. Use the following instructions to denature and dilute the 10 nM PhiX library to 8 pM. This should result in a cluster density of 750-850 K/mm².
 - ii. Combine the following volumes to dilute the PhiX library to 2 nM:
 - 10 nM PhiX library (2 µl)
 - 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20 (8 µl)
 - iii. Combine the following volumes of 2 nM PhiX library and 0.2 N NaOH in a microcentrifuge tube to result in a 1 nM PhiX library:
 - 2 nM PhiX library (10 µl)
 - 0.2 N NaOH (10 µl)
 - iv. Vortex briefly to mix the 1 nM PhiX library solution.
 - v. Briefly centrifuge the template solution.
 - vi. Incubate for five minutes at room temperature to denature the PhiX library into single strands.
 - vii. Add the following volume of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library.

- Denatured PhiX library (20 µl)
 - Pre-chilled HT1 (980 µl)
- viii. Dilute the denatured 20 pM PhiX library to 8 pM as follows:
- 20 pM denatured PhiX library (400 µl)
 - Pre-chilled HT1 (600 µl)
- d. Mix Sample Library and PhiX Control
- i. Combine the following volumes of denatured PhiX control library and your denatured sample library to result in a 15% volume ratio:
 - 8 pM PhiX control library (105 µl)
 - Denatured sample library (595 µl)
 - ii. Set the combined sample library and PhiX control aside on ice until you are ready to load it onto the MiSeq reagent cartridge.
3. Load Sample Libraries onto Cartridge
- a. Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir labeled Load Samples, position 17 on the reagent cartridge.
 - b. Pipette 700 µl of your sample libraries into the Load Samples reservoir.
4. Load Old Nextera Primer onto Cartridge.
- a. Old Nextera Primer is a custom Read 1 primer that gets spiked into position 12 on the reagent cartridge. Old Nextera Primer sequence can be found in Table 1.
 - b. In a new microcentrifuge tube, add 3.8 µl of 100 µM Old Nextera Primer, and place on ice.
 - c. Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir position 12 on the reagent cartridge.
 - d. Pipette approximately 500 µl of the Read 1 Primer Mix out of the cartridge using a 1 ml serological pipette.
 - e. Add the Read 1 Primer Mix to the tube with the Old Nextera Primer.
 - f. Vortex briefly to mix the primer solution, and then briefly centrifuge the solution.
 - g. Pipette the entire primer solution back into position 12 on the reagent cartridge using a 1 ml pipette tip.
- Note: Do not pierce any other reagent positions, they will be pierced when the cartridge is loaded on the instrument.*
- h. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface. Index sequences required for MCS can be found in Table 2.

Notes:

1. *In the Illumina Experiment Manager (the software where you create the sample sheet) there is an option to select “Custom Primer for Read 1”. Since we are spiking in the custom primer into position #12, we do NOT select that option.*
2. *Double check the primer sequence is correct and it is diluted to 100 µM. It is important that the custom primer gets mixed well with the other primers in position #12. We recommend pipetting 3.8 µl of primer into a new Eppendorf tube. Then remove the contents of position #12 and add them to*

the Eppendorf tube. Vortex to mix well. Then pipette the entire contents back into position #12 of the reagent cartridge. Tap the cartridge to make sure the liquid falls to the bottom of the tube.

3. Do **NOT** add Old Nextera Primer into Positions 18 to 20 (custom sequencing primers).
4. It is very important that the stock solution of NaOH is 10 N or higher. Some people try to use either 5 N or 2 N stock. For some reason these lower concentration stocks don't work as well for denaturation and the 0.2 N dilution has to be made on the day of loading samples on the MiSeq.
5. The denatured 20 pM PhiX library can be stored up to three weeks if stored at -15°C to -25°C. After three weeks, cluster numbers tend to decrease.

Data analysis

1. Use the Illumina bcl2fastq pipeline (v.2.20.0) for initial data processing. This pipeline will de-multiplex sequences based on their barcodes. In this experiment design, each barcoded library is a library constructed from an RNA specimen, either from the treatment group or from the control group. In this step, only the library barcodes are de-multiplexed. The region and Primer ID de-multiplexing will be done in the following steps.
2. Use TCS pipeline (v.1.3.8) to de-multiplexed each target region and create template consensus sequences. There are two ways to process it.
 - a. (Recommended) Use TCS web app (<https://tcs-dr-dept-tcs.cloudapps.unc.edu/>) to process the raw MiSeq sequences automatically. The web app asks for the information of each sequenced regions including a name, the cDNA primer sequence and the first round PCR forward primer sequence. Choose direct upload, UNC Longleaf sequence repo or Dropbox to upload the raw MiSeq sequences. The processed sequences will be returned to the email address provided by the user. The process time is from 20 min to 2 h, based on the size of files for each batch submitted.
 - b. Use the TCS pipeline script (<https://github.com/SwanstromLab/PID/blob/master/TCS.rb>) to process the MiSeq raw sequence data manually, following the instructions in the script. It is recommended to use the file-sorting and logging script in the TCS pipeline 'log_mutli.rb' (https://github.com/SwanstromLab/PID/blob/master/log_multi.rb) to sort the files after running the TCS pipeline. The script can be run on the Linux/MacOS platform using Terminal and Ruby without additional support, and in Windows platform with terminal and Ruby installed.
3. Use the 'end_join.rb' script (https://github.com/SwanstromLab/PID/blob/master/end_join.rb) in the TCS pipeline to join the paired-end sequences.
4. The number of TCS is an important parameter that reveals the sampling depth of sequencing as the number of initial templates actually queried.
5. Use the RUBY package 'viral_seq' (1.0.8) to calculate the mutation rates of each specimen. An executable script can be found in the TCS pipeline (https://github.com/SwanstromLab/PID/blob/master/mut_table.rb). This script compares each individual sequence with a sample consensus sequence and generates a table listing frequencies of each type of substitutions.

Notes:

1. *The newer version of the TCS pipeline (version $\geq 2.0.0$) is included in the RUBY package 'viral_seq' (version $\geq 1.0.8$) as an executable command line tool. It has several major updates including the end-joining functions along with the TCS creation, thus it is recommended for future use.*
2. *The authors dedicated to improve the bioinformatics analysis pipelines. Users are welcome to contact the authors for any related questions.*

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Competing interests

The University of North Carolina is pursuing IP protection for Primer ID and R.S. has received nominal royalties.

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Evaluation of the Sequence Variability within the PCR Primer/Probe Target Regions of the SARS-CoV-2 Genome

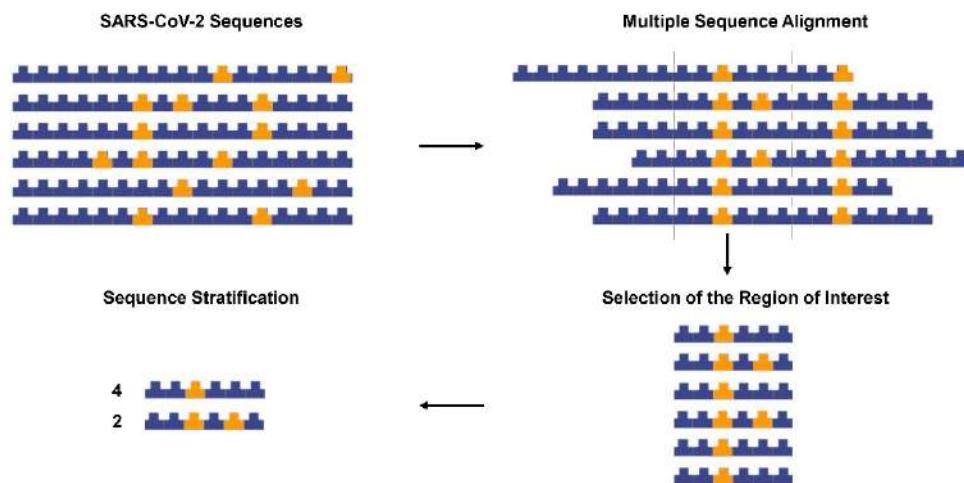
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[Abstract] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; initially named 2019-nCoV) is responsible for the recent coronavirus disease (COVID-19) pandemic, and polymerase chain reaction (PCR) is the current standard method for diagnosis from patient samples. As PCR assays are prone to sequence mismatches due to mutations in the viral genome, it is important to verify the genomic variability at primer/probe binding regions periodically. This step-by-step protocol describes a bioinformatics approach for an extensive evaluation of the sequence variability within the primer/probe target regions of the SARS-CoV-2 genome. The protocol can be applied to any molecular diagnostic assay of choice using freely available software programs and the ready-to-use multiple sequence alignment (MSA) file provided.

Graphic abstract:



Overview of the sequence tracing protocol. The figure was created using the Library of Science and Medical Illustrations from somersault18:24 licensed under a CC BY-NC-SA 4.0 license (<https://creativecommons.org/licenses/by-nc-sa/4.0/>).

Video abstract: <https://youtu.be/M1IV1iWE9k>

Keywords: Coronavirus SARS-CoV-2, COVID-19, Diagnosis, Genomic variability, Polymerase chain reaction (PCR), Mutations

[Background] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; initially named as 2019-nCoV) is the cause of novel coronavirus disease termed COVID-19. The virus originated from Wuhan, China.

China and rapidly spreaded around the world causing a global pandemic (Worldometers.info, 2020). Sequencing of the virus showed that its single-stranded RNA genome is ~30 kb in size (Chan *et al.*, 2020; Lu *et al.*, 2020; Wu *et al.*, 2020; Zhou *et al.*, 2020). Availability of the viral sequence early in the outbreak helped the development of several polymerase chain reaction (PCR) detection protocols that have been instrumental in the diagnosis of the disease from patients samples (WHO, 2020). However, genetic variability in the viral genome during natural evolution poses a potential risk of mismatches between the diagnostic assays and the template that can result in false-negative results (Whiley and Sloots, 2005; Chow *et al.*, 2011). Sequences of SARS-CoV-2 viruses isolated from around the world are being deposited in the sequence databases and mutations have been identified in the genomes of the circulating viruses (Ugurel *et al.*, 2020).

We performed an extensive evaluation of published diagnostic PCR assays, including those recommended by the World Health Organization (WHO), based on evaluation of sequence variation in the primer/probe binding regions using more than 17,000 publicly available viral sequences (Khan and Cheung, 2020). Another concurrent publication reported mutations in primer/probe binding regions using 1825 sequences but a detailed sequence tracing protocol was not provided (Osorio and Correia-Neves, 2020). This step-by-step protocol outlines a bioinformatics pipeline that uses freely available open-source software programs. The pipeline can be performed on a regular desktop computer without any need for special hardware and does not require extensive computational skills. The provision of a ready-to-use Multiple Sequence Alignment (MSA) file through Open Science Framework (OSF) makes it an even more intuitive task. Inclusivity analysis through verification of *in silico* nucleotide identity match is one of the regulatory requirements for approval of COVID-19 diagnostic assays (Commission-Services, 2020; FDA, 2020; Health-Canada, 2020). The protocol can also be applied to other molecular diagnostic assays of SARS-CoV-2 including point-of-care CRISPR-based diagnostic assays under development (Tsang and LaManna, 2020).

Equipment

1. A regular Windows or Mac OS X laptop or desktop.

Note: There is no specific processor or RAM requirement, but memory issues can be avoided by opening a limited number of files at the same time. The outlined protocol was performed on a laptop installed with Windows 10, an Intel Core i5-8265U processor, CPU @1.60GHz and an 8 GB RAM.

Software

1. MAFFT version 7 online service (Katoh *et al.*, 2002 and 2019) (available from <https://mafft.cbrc.jp/alignment/software/closelyrelatedviralgenomes.html>)
2. AliView version 1.26 (Larsson, 2014) (available from <https://ormbunkar.se/aliveview/>)
3. Sequence Manipulation Suite version 2 (Stothard, 2000) (available from https://www.bioinformatics.org/sms2/rev_comp.html)
4. SequenceTracer (Nagy *et al.*, 2019) (available from

-
5. ElimDuplicates (<https://www.hiv.lanl.gov/content/sequence/elimduplicatesv2/elimduplicates.html>)
 6. PNNS calculator (available from <http://entropy.szu.cz:8080/EntropyCalcWeb/pnns>)
 7. A web browser (for example Google Chrome or Mozilla Firefox)
 8. A text editor (for example Microsoft Notepad).

Procedure

You can jump to Procedure D to download the latest version of a ready-to-use SARS-CoV-2 Multiple Sequence Alignment (MSA) file from our project page on OSF.

A. Viral sequence dataset

1. Download the viral sequences from the repository of your choice.

Note: Check the terms and conditions of each repository with attention to the data sharing policy.

Registration may be required.

- a. GISAID's EpiCoV database (<https://www.gisaid.org/>) (Shu and McCauley, 2017).
 - b. NCBI virus (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>) (Hatcher *et al.*, 2017).
 - c. The Chinese National Genomics Data Center (NGDC) database (<https://bigd.big.ac.cn/ncov>) (NGDC, 2020).
 - d. EMBL-EBI's COVID-19 Data Portal (<https://www.covid19dataportal.org/>).
 - e. COVID-19 Genomics UK (COG-UK) Consortium (<https://www.cogconsortium.uk/data/>).
2. Download the complete genome of Wuhan-Hu-1 (NCBI Reference Sequence: NC_045512.2; <https://www.ncbi.nlm.nih.gov/nucleotide/>).

B. Multiple Sequence Alignment (MSA) using MAFFT online service dedicated to MSA of closely-related viral genomes (<https://mafft.cbrc.jp/alignment/software/closelyrelatedviralgenomes.html>).

1. Input (Figure 1A):

- a. The complete genome of Wuhan-Hu-1 (NC_045512.2) to the "Existing alignment" box.
- b. Input the other sequences to the "Fragmentary sequence(s)" box.

Note: MAFFT online service supports up to 20,000 sequences of ~30 kb in length. The task should be performed in batches if more sequences are being aligned and results should be combined after sequence stratification.

2. Parameters (Figure 1B):

- a. UPPERCASE/lowercase, select "same as input".
- b. Direction of nucleotide sequences, select "Adjust direction according to the first sequence".
- c. Output order, select "aligned".

3. Advanced Settings (Figure 1C):

- a. Keep alignment length, select "Yes".
- b. Strategy, select "auto".

4. Download the aligned sequence in FASTA format, once available.

A

Existing alignment: [Example](#)
Gaps (-) will be preserved.

or upload a plain text file: No file chosen
Zipped file is acceptable.

B

UPPERCASE / lowercase:
 Same as input
 Amino acid – UPPERCASE / Nucleotide – lowercase

Direction of nucleotide sequences:
 Same as input
 Adjust direction according to the first sequence accurate enough for most cases! [Help](#)
 Adjust direction according to the first sequence (only for highly divergent data, very slow) [Help](#)

Output order:
 Same as input
 Insert "New" at the head of title of each new sequence
 Aligned

Sequence title:
 Same as input
 Insert "New" at the head of title of each new sequence

Job name (optional): (basic Latin alphabet, number and space only)

Notify when finished (optional, recommended when submitting large data):
 Email address

C

Advanced settings
Keep alignment length in [Aligner setting \(2015-Nov\)](#)
 Yes
With this option, whenever all the **Fragmentary sequence(s)** are defined, it will keep the alignment length the same as the target alignment.

A **SEQUENCE ALIAS** between the positions in each **Fragmentary sequence** and this positions in the alignment will also be retained.

Targets:
 Align to: [Target sequence](#) (keep your desired sequence)
 Reverse (R)
 Inverse (W)
 Inverse (W) + Inverse (R)
 Inverse (R) + Inverse (W)

Figure 1. Multiple Sequence Alignment (MSA) using MAFFT online service

Video for Procedure A-B: <https://youtu.be/hbnsXnikRak>

- C. Alternatively, download a ready-to-use MSA file from our OSF page (<https://doi.org/10.17605/OSF.IO/NPCS6>).

Note: Data in our original publication (Khan and Cheung, 2020) was downloaded from GISAID that does not permit the release of MSA files publicly. The MSA file provided on our OSF page was generated using sequences downloaded from the NCBI virus. The file would be updated periodically during the pandemic (file 1 aligning 19863 SARS-CoV-2 sequences).

- D. Save Region of Interest (ROI) for each primer/probe as a separate FASTA file

1. Open the MSA file from Procedure B or Procedure C in the AliView program.

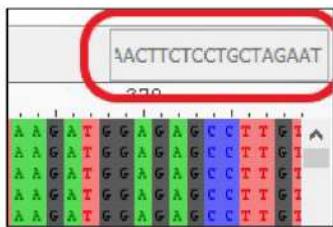
Note: Aliview program (available from <https://ombunkar.se/aliview/>) needs to be downloaded on the computer in advance.

2. Find the primer binding site using the “find” function (Figure 2A) or using “add and align sequences from clipboard” function (Figure 2B).
3. Reverse-complement the primer/probe sequence as necessary using Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/rev_comp.html).

>CN-CDC-N_F

GGGGAACTTCTCCTGCTAGAAT

A



B

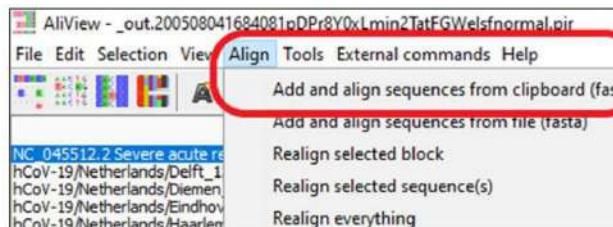
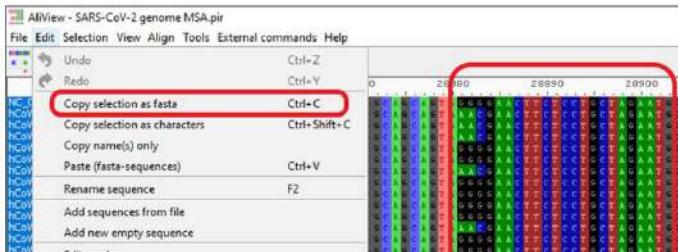


Figure 2. Finding the Region of Interest (ROI) in MSA

4. Select the ROI and copy selection as FASTA format (Figure 3A).
5. Open a new file, "Paste (fasta-sequences)" and "Save" (Figure 3B). The sequence can be pasted in a text editor and saved as a FASTA file.

A



B



Figure 3. Saving Region of Interest (ROI) for each primer/probe as a separate FASTA file

Video for Procedure D: <https://youtu.be/H9UxkgAsMdE>

E. Sequence stratification: Option 1 – SequenceTracer

1. Upload the individual FASTA file to SequenceTracer and hit "Submit" (Figure 4A) (<http://www1.szu.cz:8080/EntropyCalcWeb/sequences>). The SequenceTracer segregates data into discrete groups of identical sequence variants and presents a detailed view of the nucleotide variation in each ROI along with the frequency of each variant. Moreover, the sequences showing ambiguous sequences are grouped as "outgroup1", short sequences are grouped as "outgroup2" and missing sequences are grouped as "excluded".
2. Download the stratified data showing a list of sequence variants and/or a chart (Figure 4B).

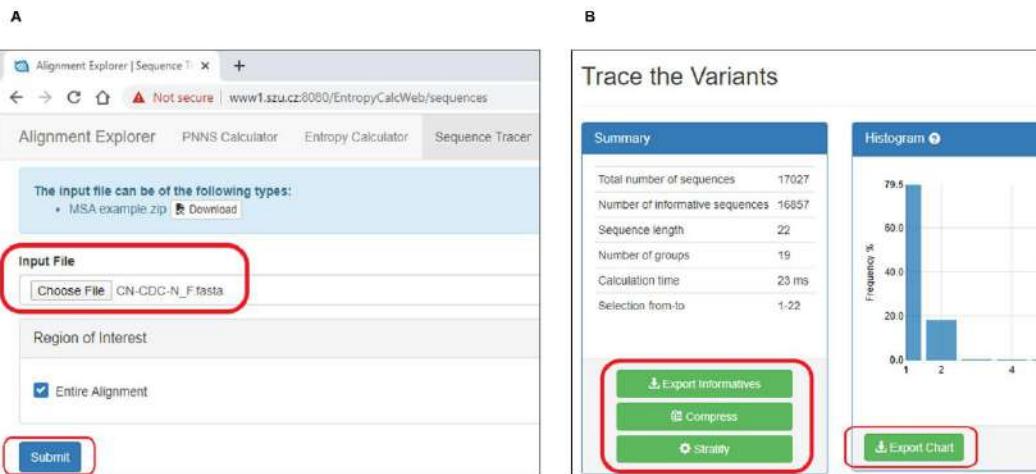


Figure 4. Sequence stratification using SequenceTracer

Video for Steps E1-E2: https://youtu.be/ysT_KBXkpvw

3. The "stratify" file can be opened using Microsoft Excel while the "compressed" file can be opened using the AliView program or a text editor (Figure 5).
4. The data of any sequence variant group can be downloaded (Figure 6).
 - a. First, select the group.
 - b. Then, "Add all to Notes".
 - c. Finally, "export".

Group Number	Variant Count	Frequency %	10	20
			
			GGGGAACCTCTCCTGCTAGAAT	
1	13533	79.480
2	3129	18.377	aac.....
3	85	0.499	g.....
4	48	0.282t.....
5	22	0.129	c....
6	9	0.053	a.....
7	4	0.023t.....
8	4	0.023	c.....
9	4	0.023	t....
10	3	0.018	t.....
11	3	0.018	.t.....
12	3	0.018	..a.....
13	3	0.018	aac...t.....
14	2	0.012	a...
15	1	0.006	t.....
16	1	0.006c.....
17	1	0.006g.....
18	1	0.006	aa.....
19	1	0.006	aac.....t.....
outgroup1	167	0.981		
outgroup2	3	0.018		

Figure 5. Expected results from SequenceTracer

The screenshot shows a 'Groups' table and a 'Group Details' table. In the 'Groups' table, the second row (Group Number 2) is highlighted with a red box. In the 'Group Details' table, the second row (Sequence Number 2) is also highlighted with a red box. Below these tables are 'Add all to Notes' and 'Remove All' buttons. At the bottom are 'Remove All' and 'Export' buttons. The 'Export' button is highlighted with a red box.

Group Number	Variant Count	Frequency
1	13533	79.40%
2	3126	18.37%
3	63	0.489%
4	48	0.281%
5	22	0.129%
6	8	0.053%
7	4	0.023%
8	4	0.023%
9	4	0.023%
10	3	0.018%
11	3	0.018%
12	3	0.018%
13	3	0.018%
14	2	0.012%
15	1	0.008%
16	1	0.008%
17	1	0.008%
18	1	0.008%
19	1	0.008%
outgroup1	187	0.081%

Group Number	Sequence Number	Sequence
-	2	>hCoV-19/Netherlands/Delft_1383424/2020 EPI_ISL_413589 2020-02-28
-	3	>hCoV-19/Netherlands/Diemen_1383454/2020 EPI_ISL_413570 2020-02-28
-	4	>hCoV-19/Netherlands/Eindhoven_1383782/2020 EPI_ISL_413571 2020-03-02
-	7	>hCoV-19/Netherlands/Helmond_1383548/2020 EPI_ISL_413574 2020-02-29
-	12	>hCoV-19/Netherlands/Noordorp_1384222/2020 EPI_ISL_413579 2020-03-03
-	17	>hCoV-19/Netherlands/Rotterdam_1304740/2020 EPI_ISL_413584 2020-03-03

Figure 6. Downloading data of a specific variant group

Video for Steps E3-E4: <https://www.youtube.com/watch?v=4S0T9TW5ax4>

F. Sequence stratification: Option 2 – ElimDuplicates

1. Upload the individual FASTA file to ElimDuplicates (<https://www.hiv.lanl.gov/content/sequence/elimduplicatesv2/elimduplicates.html>).
2. Select parameters as shown and hit "Submit" (Figure 7A).
3. View or Download "Unique sequences with rank and count appended (_count)" (Figure 8B).
4. The file would show sequence variants, along with rank and count added to the sequence name. The file can be opened using the AliView program or a text editor (Figure 7C).

Note: As opposed to SequenceTracer, variants with ambiguous sequences, and with missing sequences would be ranked in the results and need to be separated manually.

A: The 'Input' section shows a text area for pasting sequences and a 'Choose File' button. Below it are checkboxes for sequence alignment and grouping. The 'Elimination options' section includes settings for sequence elimination (100% identical or >80% similar), removing extraneous characters, making all letters uppercase, and considering subsequences as duplicates. The 'Output options' section allows restoring original sequences, creating unique sequence files with counts, and including rank in sequence names. Buttons for 'Submit' and 'Reset' are at the bottom.

B: The 'Unique sequences with rank and count appended (_count)': View Download button is highlighted with a red box. Below it are links for 'Unique sequences file: View Downloaded', 'Duplicate (eliminated) sequence file: View Downloaded', and a 'Tab-delimited summary table below: View Downloaded'.

C: The 'Download this file' section shows a list of sequence variants. One variant is highlighted with a red box: '>hCoV-19/USA/TN-1000413/2020|EPI_ISL_433006|2020-04-21_8_45 CCCGAACTTCTCCCTGTAGAT'. The 'Rank & Count' column is also highlighted with a red box.

Figure 7. Sequence stratification using ElimDuplicates

G. Position Nucleotide Numerical Summary (PNNS)

- As the sequence variation was moderate, the base composition of each nucleotide position was not analyzed in the original publication. This can be performed for highly variable regions using the Position Nucleotide Numerical Summary (PNNS) calculator (<http://entropy.szu.cz:8080/EntropyCalcWeb/pnns>).

Data analysis

First, the sequences with ambiguous nucleotides (outgroup 1), short sequences (outgroup 2) and missing sequences (excluded) are removed and the number of “informative” sequences is calculated by subtracting these three groups from the total number of sequences. SequenceTracer performs this calculation automatically whereas the calculation needs to be performed manually if using ElimDuplicates. The informative group is then divided into hits with a perfect match and hits with mismatches for each primer and probe. To minimize the effect of low prevalent variants and sequencing errors in the data on the analysis, we defined a threshold of 0.5% in our original publication (Khan and Cheung, 2020) where only the sequence variants with $\geq 0.5\%$ incidence were further considered. As more high quality viral sequences become available a more stringent threshold (for instance 0.1%) may be defined. Another way of defining a threshold is to include all the mutations in the analysis that occur more than once in different sequencing experiments (Osorio and Correia-Neves, 2020). The number and frequency of the sequences with the perfect match and with mismatches are then calculated from sequences above the defined threshold for each primer and probe. As an example, the analysis of CN-CDC-N forward primer 5'-GGGAACTTCTCCTGCTAGAAT-3' (WHO, 2020) is shown in Table 1. The summary of the analysis for 27 previously published PCR assays is presented in Table 2 of our previous publication (Khan and Cheung, 2020).

Mismatches can also be divided into mismatches in the 3' end (last 5 nucleotides) and the 5' end. It is known that PCR amplification is more prone to mismatches at the 3' end of the primer (Whiley and Sloots, 2005; Stadhouders *et al.*, 2010; Lefever *et al.*, 2013). Moreover, mismatches in the probe can have a deleterious effect on PCR amplification. Even a single mismatch may reduce the sensitivity of the assay and lead to false-negative results due to the prevention of probe binding and subsequent fluorescence (Chow *et al.*, 2011; Brault *et al.*, 2012).

Table 1. Analysis of CN-CDC-N forward primer

		Number of sequences (frequency)
Total number of sequences		17027
Removed sequences (outgroup1 + outgroup2 + excluded)		170
Informative sequences		16857
Sequences with the perfect match		13533
Sequences with mismatches		3324
Sequences above threshold (0.5%)	Total	16662
	Perfect match	13533 (81.22%)
	With mismatches	3129 (18.78%)
Sequences above threshold (0.1%)	Total	16817
	Perfect match	13533 (80.47%)
	With mismatches	3284 (19.53%)
Sequences with an occurrence of more than 1	Total	16852
	Perfect match	13533 (80.31%)
	With mismatches	3319 (19.69%)

Acknowledgments

The protocol is the detailed version of the method used in our previous publication (Khan and Cheung, 2020). We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiCoV™ Database on which our research is based. The list is included in electronic supplementary material, file 1 of our original publication. Funding for this study was provided by the Canadian Institutes of Health Research operating grant (number RN227427 – 324983) awarded to PC.

Competing interests

The author has no competing interests.

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Computational Analysis and Phylogenetic Clustering of SARS-CoV-2 Genomes

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[Abstract] COVID-19, the disease caused by the novel SARS-CoV-2 coronavirus, originated as an isolated outbreak in the Hubei province of China but soon created a global pandemic and is now a major threat to healthcare systems worldwide. Following the rapid human-to-human transmission of the infection, institutes around the world have made efforts to generate genome sequence data for the virus. With thousands of genome sequences for SARS-CoV-2 now available in the public domain, it is possible to analyze the sequences and gain a deeper understanding of the disease, its origin, and its epidemiology. Phylogenetic analysis is a potentially powerful tool for tracking the transmission pattern of the virus with a view to aiding identification of potential interventions. Toward this goal, we have created a comprehensive protocol for the analysis and phylogenetic clustering of SARS-CoV-2 genomes using Nextstrain, a powerful open-source tool for the real-time interactive visualization of genome sequencing data. Approaches to focus the phylogenetic clustering analysis on a particular region of interest are detailed in this protocol.

Keywords: COVID-19, SARS-CoV-2, Phylogenetic analysis, Genomes, Coronavirus

[Background] Severe Acute Respiratory Syndrome- related coronaviruses (SARS-CoV) are one of the largest single-stranded RNA virus families known to date (Zhu *et al.*, 2020). Recently, SARS-CoV-2, a novel strain of coronavirus, has been identified as the causal pathogen for the ongoing Coronavirus disease 2019 (COVID-19) pandemic (Huang *et al.*, 2020). The infectious disease that first originated in Wuhan, China, spread to other nations at an alarmingly rapid pace. With 3,517,345 cases reported globally and a death toll of 243,401 (as of 5th May 2020), the disease continues to be a public health concern and a potential threat to the socio-economic welfare of nations and healthcare systems worldwide (World Health Organization, 2020. Novel Coronavirus (2019-nCoV): situation report, 106).

Owing to the rapid advancement of next-generation sequencing (NGS) technology and analysis methods, sequencing the viral genome has been recognized as a viable tool to aid the diagnosis and treatment of COVID-19 and help to understand the disease epidemiology. As the disease evolves over time, more sequencing data for SARS-CoV-2 genomes is being made available in the public domain. To date, there are over 25,000 publicly available genomes of SARS-CoV-2 from different geographical origins. Phylogenetic principles have previously been successfully utilized to contain and diffuse recent pandemic events such as avian influenza, the Zika virus epidemic, and HIV (Salemi *et al.*, 2008; Babakir-Mina *et al.*, 2009; Angeletti *et al.*, 2016). With the rapid accumulation of sequencing data, phylogenetic and phylodynamic analysis are potentially powerful tools for studying the evolutionary patterns of rapidly evolving RNA viruses, and therefore help to understand the epidemiology of the outbreak.

Visualizing evolutionary epidemiology can help to provide a deeper understanding of the global diversity

of SARS-CoV-2. Nextstrain is an open-source project that aims to provide real-time interactive visualization of rapidly evolving pathogens coupled with additional data such as geographic information (Hadfield *et al.*, 2018). Nextstrain utilizes Augur, a bioinformatics toolkit for the systematic analysis of genome sequences, and Auspice, an interactive web service for the visualization of analysis results. This protocol has been created to aid bioinformaticians in gaining an epidemiological understanding of the SARS-CoV-2 pathogen using the powerful phylogenetic analysis toolkit provided by Nextstrain. The data and parameters used in this protocol are specific to SARS-CoV-2 genomes; however, Nextstrain is a generalized toolkit for the analysis of pathogen phylogenies and can be customized using the appropriate data and parameters suited to the pathogen of interest. All software and datasets used in this protocol are available in the public domain.

Equipment

We explicitly assume that the user has some experience working with shell commands on a Linux-based operating system and has superuser privileges.

1. Computational Requirements

We recommend using a workstation or a server with a 64 bit Linux-based operating system, possessing 8 GB RAM and sufficient hard disk space (at least 250 GB) to store the files used and produced in this analysis. The commands given in this analysis protocol have been validated on Ubuntu (18.04 LTS) Linux Distribution.

Software

1. Required Software

This protocol uses the following tools and Nextstrain software to perform the phylogenetic analysis:

- a. Docker Engine (<https://www.docker.com/>)
- b. Anaconda (<https://www.anaconda.com/>)
- c. Nextstrain (Hadfield *et al.*, 2018)
- d. Augur (Hadfield *et al.*, 2018)
- e. MAFFT (Katoh and Standley, 2013)
- f. IQTREE (Nguyen *et al.*, 2015)

All requisite tools and their dependents must be installed before proceeding with the analysis.

2. Datasets

The protocol uses the SARS-CoV-2 genome sequence datasets made available by the Global Initiative on Sharing All Influenza Data (GISAID) (Shu and McCauley, 2017).

The installation steps for all tools used in this protocol and the instructions for downloading the requisite datasets are given in the following section.

Procedure

The individual steps involved in this protocol and the Augur modules used in each step are summarized in

Figure 1.

Downloading and installing requisite software tools and datasets

A. Install Docker Engine

Docker is an open-source technology based on virtualization, which is used for developing and running software applications in the form of containers. The Docker Engine can be installed using the following commands:

```
sudo apt-get update  
sudo apt-get install apt-transport-https ca-certificates curl gnupg-agent  
software-properties-common  
curl -fsSL https://download.docker.com/linux/ubuntu/gpg | sudo apt-key add  
-  
sudo apt-key fingerprint 0EBFCD88  
sudo add-apt-repository "deb [arch=amd64]  
https://download.docker.com/linux/ubuntu $(lsb_release -cs) stable"  
sudo apt-get update  
sudo apt-get install docker-ce docker-ce-cli containerd.io
```

To activate and test Docker installation, execute the following commands:

```
sudo groupadd docker  
sudo usermod -aG docker $USER  
newgrp docker  
docker run hello-world
```

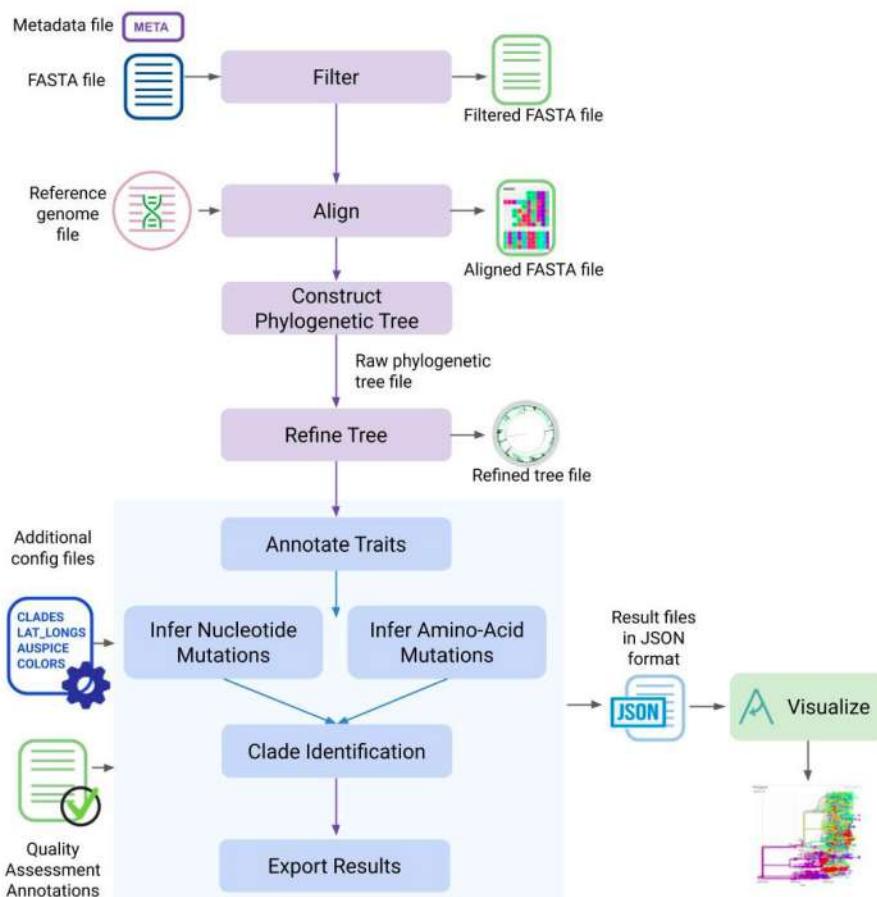


Figure 1. The different steps described in this protocol and the Augur modules used in each of the analysis steps

B. Install Anaconda

Anaconda is an open-source distribution of Python that simplifies the management of Python packages and environments. To install Anaconda, use the following commands:

```
wget https://repo.anaconda.com/archive/Anaconda3-2020.02-Linux-x86\_64.sh
bash Anaconda3-2020.02-Linux-x86_64.sh
```

Proceed with the installation by following the on-screen instructions. You can find the anaconda3 folder in the directory shown in the installer script. You can activate and test your installation by running the following commands:

```
source ~/.bashrc
conda list
```

C. Install Nextstrain-CLI

Nextstrain is available as a Python package and can be installed using pip.

```
python3 -m pip install nextstrain-cli
```

To check whether Nextstrain has been successfully installed, use the following command:

```
nextstrain version
```

The version number shown in the output should be 1.16.1 or higher.

D. Install Augur

Augur is the toolkit provided by Nextstrain for phylogenetic analysis. Augur is also available as a Python package and can be installed using the following command:

```
python3 -m pip install nextstrain-augur
```

E. Install MAFFT

MAFFT (Multiple Alignment using Fast Fourier Transform) is required by Augur to perform multiple-sequence alignments. To download and install this tool, use the following command:

```
sudo apt-get install mafft
```

F. Install IQ-TREE

IQ-TREE is an open-source tool for constructing maximum-likelihood trees using phylogenetic data. IQ-TREE is required by Augur for constructing a phylogenetic tree from sequence data. To install IQ-TREE use the following command:

```
sudo apt-get install iqtreetree
```

It is recommended to use IQ-TREE version 1.6.1 (default version installed for Ubuntu 18.04 LTS) or higher.

G. Download the SARS-CoV-2 sequence dataset

The Global Initiative on Sharing All Influenza Data (GISAID) is the most updated public repository of SARS-CoV-2 genome sequences. For this phylogenetic clustering protocol, we downloaded the dataset of ~15,000 complete (as of 1st May 2020) SARS-CoV-2 genome sequences from GISAID. The database can be accessed by registering for a GISAID account. Upon successful activation, the sequence dataset can be downloaded by logging into the GISAID EpiCoV™ database and navigating to the Browse option (<https://www.epicov.org/epi3/frontend>).

To create the metadata file required by Augur, you will also need to download the Acknowledgment Table for all submissions provided by GISAID, which can also be found on the Browse page.

H. Download the SARS-CoV-2 reference genome

Before proceeding with the analysis, you also need to download the reference genome for SARS-CoV-2 from NCBI in GenBank (.gb). For this analysis, we downloaded the genome with the accession number MN908947.3.

I. Preparing input files

To use Nextstrain for phylogenetic analysis and visualization, you need to prepare the following input files (Table 1):

Table 1. List of input files required to run the different steps in the analysis pipeline

File	Description
Required Input Files	
sequences.fasta	Collection of SARS-CoV-2 sequences to be analyzed in FASTA format
metadata.tsv	Tab-delimited text file describing all sequences in the sequences.fasta file
clades.tsv	Tab-delimited text file containing clade definitions downloaded from the Nextstrain GitHub repository
MN908947.gb	SARS-CoV-2 reference genome in Genbank format
Additional Configuration Files	
auspice_config.json	Text file in JSON format specifying visualization settings
lat_long.tsv	Tab-delimited text file for displaying geographic traits
colors.tsv	Tab-delimited file containing hex colour codes for metadata elements
Optional Configuration Files	
include_file	Text file containing names of sequences to be included in the analysis regardless of other subsampling criteria

1. sequences.fasta

A single FASTA file containing a collection of pathogen sequences to be analyzed. For this analysis, we used the sequence dataset downloaded from GISAID. Each sequence in the FASTA file should have the strain ID of the virus as the sequence header. A sample sequence record for the FASTA file is shown in Figure 2.

```
>hCoV-19/India/1-27/2020
ACCTTCCCAGGTAAACAAACCAACCAACTTCGATCTCTTAGATCTGTTCTCTAAACGA
ACTTAAAATCTGTGTGGCTGTCACTCGGCTGCATGCTTAGTGCACTCACGCAGTATAAT
TAATAACTAATTACTGTCGTTGACAGGACACGAGTAACCTCGTCTATCTTCTGCAGGCTGC
TTACGGTTCGTCCGTGTTGCAGCCGATCATCAGCACATCTAGGTTCGTCCGGGTGTGA
CGAAAGGTAAGATGGAGAGGCCCTGTCCTGGTTCAACGAGAAAACACACGTCCAACTC
AGTTGCCTGTTTACAGGTTCGCGACGTGCTGTACGTGGCTTGAGACTCCGTGGAG
GAGGTCTTATCAGAGGCACGTCAACATCTAAAGATGGCACTTGTGGCTTAGAAGTT
GAAAAAGGCCTTGCCTCAACTTGAACAGCCCTATGTGTTCATCAAACGTTCGGATGCT
CGAACTGCACCTCATGGTCATGTTATGGTTGAGCTGGTAGCAGAACCTCGAAGGCATTAG
TACGGTCGTAGTGGTGGAGACACTTGGTGCCTGTCCTCATGTGGCGAAATACCAGTG
GCTTACCGCAAGGTTCTTCTCGTAAGAACGGTAATAAAGGAGCTGGTGGCCATAGTTAC
GGCGCCGATCTAAAGTCATTGACTTAGGCAGCAGCTGGCACTGATCCTTATGAAGAT
TTCAAGAAAACCTGGAACACTAAACATAGCAGTGGTGTACCGTGAACCTCATGCGTGAG
CTAACCGGAGGGGCATACACTCGCTATGTCGATAACAACCTCTGTGGCCCTGATGGCTAC
CCTTGTGAGTCATTAAAGACCTCTAGCACGTGCTGGTAAAGCTTCATGCACTTGTCC
```

Figure 2. Sample record for the hCoV-19/India/1-27/2020 SARS-CoV2 strain in the sequences.fasta format

2. metadata.tsv

A tab-delimited metadata file that describes the sequences given in the FASTA file. The various fields to be included in the metadata file are as follows:

a. Required fields: Strain, Virus, Date

For each strain ID in the sequences.fasta file, there should be an entry under the strain column in the metadata file.

b. Additional fields (if using published data): Accession, Authors, URL, Title, Journal, Paper_URL.

c. To infer ancestral traits, additional information fields such as region, country, state, and city need to be included in the metadata file.

The information for the various fields in the metadata file can be taken from the Acknowledgment Table downloaded from GISAID. A sample metadata spreadsheet is linked here as [Supplementary Data 1](#).

3. clades.tsv

This file is required for the addition of clade labeling to the phylogenetic tree. The file specifies the mutations (amino acid or nucleotide) specific to a particular clade of the virus (Figure 3). The clades.tsv file should contain the following fields:

a. clade: To describe the name of a clade.

b. gene: The name of the gene in which the mutation lies (for nucleotide changes, the gene name should be ‘nuc’).

c. site: The position of the mutation within the genome.

d. alt: The mutated amino acid or nucleotide found at that position.

For this analysis, we used the clades definition for SARS-CoV-2 genomes defined by Nextstrain (<https://github.com/nextstrain/ncov>).

clade	gene	site	alt
A1a	ORF3a	251	V
A1a	ORF1a	3606	F
A2	S	614	G
A2a	ORF1b	314	L
A3	ORF1a	378	I
A3	ORF1a	3606	F
A6	nuc	514	C
A7	ORF1a	3220	V
B	ORF8	84	S
B1	ORF8	84	S
B1	nuc	18060	T
B2	ORF8	84	S
B2	nuc	29095	T
B4	ORF8	84	S
B4	N	202	N
B4	N	202	N

Figure 3. Summary screenshot of the clades.tsv file provided by Nextstrain for SARS-CoV-2 genomes

4. auspice_config.json

This file is needed to set various display options for visualization. A sample config file is linked here as [Supplementary Data 2](#).

5. lat_long.tsv

A tab-separated file containing latitudes and longitudes for all regions, countries, states, and cities in the dataset (Figure 4). This file will be used to display geographic traits during visualization.

state	Odisha	20.5431241	84.6897321
state	Punjab	30.9293211	75.5004841
state	Tamil Nadu	10.9094334	78.3665347
city	New Delhi	28.704060	77.102493
city	Ahmedabad	23.0216238	72.5797068
city	Gandhinagar	23.2232877	72.6492267
city	Mansa	23.64955612	72.29003906
city	Jaipur	26.916194	75.820349
city	Mumbai	18.9387711	72.8353355
state	Jammu and Kashmir	33.53155445	75.04417419
state	Assam	26.4073841	93.2551303
state	Madhya Pradesh	23.9699282	79.39486955
city	Surat	21.19177615	72.95441578
city	South 24 Parganas	22.1815262	88.53780484
city	Howrah	22.4993915	88.03091255
city	Prantij	23.39009355	72.83209868
city	Modasa	23.4634245	73.2990631
city	Dhansura	23.4634245	73.2990631

Figure 4. Summary screenshot of the lat_long.tsv file required by Nextstrain for visualizing geographic traits

6. Quality assessment

In this visualization, we would also like to segregate high-quality FASTA sequences in the dataset from low-quality ones. Accordingly, we added an additional field, ‘quality,’ to the metadata file. The

following quality metrics define a high-quality sequence:

- a. Percentage identity to the reference genome after pairwise alignment: >99%
- b. Percentage of gaps in the alignment: <1%
- c. Percentage of N (unknown nucleic acid residue) bases in the sequence: <1%
- d. No degenerate bases in the sequence

Based on the above criteria, the ‘quality’ metadata field can hold the values, ‘High,’ ‘Low,’ and ‘Not Assessed.’

To visualize the quality assessment, we created an additional configuration file ‘colors.tsv,’ a tab-delimited file containing hex codes for each value of the sequence quality field that you want to represent. In this analysis, high-quality is shown in green, low-quality in red, and unassessed sequences in yellow by specifying the corresponding hex codes for the required colors in the ‘colors.tsv’ file (Figure 5).

```
quality Low      #FF0000
quality High     #50C878
quality Not Assessed #FFFF9C
```

Figure 5. Summary screenshot of the colors.tsv file created for visualizing sequence quality

Data analysis

Due to legibility and performance constraints, Nextstrain can only handle ~3,000 sequences in a single view. Since we are working with a set of ~15,000 genome sequences, we subsampled our data and analyzed them by focusing on an individual geographic region (*i.e.*, India).

A. Filter sequences

The input sequence set can be filtered based on certain criteria and subsampled using this command. The following command will filter the SARS-CoV2 sequences based on their submission dates and group them by country, year, and month. All sequences dated prior to 2013 or possessing a missing date record will be dropped. The global data will also be subsampled to 100 sequences per country per year per month.

```
augur filter --sequences <sequences.fasta> --metadata <metadata.tsv> --
output <filtered_ncov.fasta> --group-by country year month --sequences-per-
group 100 --min-date 2013
```

To focus on a particular geographic region, the filter command also contains parameters that help to include or exclude certain sequences from the analysis:

```
--include <include_file> This constraint can be used to include sequences
```

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regardless of other subsampling criteria. For this analysis, the `include_file` will contain the line `hCoV-19/Wuhan/WH01/2019`, since we will be using this genome as the root in the phylogenetic tree. The names of any other sequences that you want to include in your analysis can be added to this file.

`--exclude-where <CONDITION>` This constraint will be used for focusing the analysis on a particular region.

To subsample the dataset for a single geographic region, use the following command:

```
augur filter --sequences <sequences.fasta> --metadata <metadata.tsv> --  
output <filtered_ncov_india.fasta> --exclude-where country!=India --include  
<include_file>
```

B. Alignment to the reference genome

Augur uses MAFFT to perform multiple-sequence alignments. To create an alignment file using Augur use the following command:

```
augur align --sequences <filtered_ncov.fasta> --reference-sequence  
<MN908947.gb> --output <aligned_ncov.fasta> --nthreads <2> --remove-  
reference --fill-gaps
```

For the geographic region-focused analysis, use the following command:

```
augur align --sequences <filtered_ncov_india.fasta> --reference-sequence  
<MN908947.gb> --output <aligned_ncov_india.fasta> --nthreads <2> --remove-  
reference --fill-gaps
```

C. Constructing the phylogenetic tree

Augur uses IQTREE as the default software to construct a phylogenetic tree from the multiple-sequence alignment file. The branch lengths in the tree are a measure of nucleotide divergence. The following command will generate a phylogenetic tree in Newick format (.nwk):

```
augur tree --alignment <aligned_ncov.fasta> --output <raw_tree_ncov.nwk> --  
nthreads <4>
```

For the geographic region-focused analysis, use the following command:

```
augur tree --alignment <aligned_ncov_india.fasta> --output  
<raw_tree_ncov_india.nwk> --nthreads <4>
```

D. Refining the phylogenetic tree

The raw tree constructed in the previous step can be further processed by Augur using TreeTime to Copyright © 2021 The Authors; exclusive licensee Bio-protocol LLC.

adjust the branch lengths according to the sampling dates of the sequences. For this analysis, we specified the root of the tree by giving the sequence name *hCoV-19/Wuhan/WH01/2019* explicitly with the *--root* parameter of the *refine* command. The *--clock-rate* parameter was used to run the analysis using a fixed evolutionary rate to produce a robust time-resolved phylogeny, and the *--clock-filter-iqd* parameter filters out genomes that do not follow the evolutionary rate or molecular clock. For SARS-CoV-2 genomes, this rate is fixed at 0.0008 or 8×10^{-4} substitutions per site per year. To produce a time-resolved tree use the following command:

```
augur refine --tree <raw_tree_ncov.nwk> --alignment <aligned_ncov.fasta> --  
metadata <metadata.tsv> --output-tree <refined_ncov_tree.nwk> --output-  
node-data <branch_lengths_ncov.json> --root hCoV-19/Wuhan/WH01/2019 --  
timetree --clock-rate 0.0008 --clock-std-dev 0.0004 --coalescent skyline --  
date-inference marginal --divergence-unit mutations --date-confidence --no-  
covariance --clock-filter-iqd 4
```

For the geographic region-focused analysis, use the following command:

```
augur refine --tree <raw_tree_ncov_india.nwk> --alignment  
<aligned_ncov_india.fasta> --metadata <metadata.tsv> --output-tree  
<refined_ncov_tree_india.nwk> --output-node-data  
<branch_lengths_ncov_india.json> --root hCoV-19/Wuhan/WH01/2019 --timetree  
--clock-rate 0.0008 --clock-std-dev 0.0004 --coalescent skyline --date-  
inference marginal --divergence-unit mutations --date-confidence --no-  
covariance --clock-filter-iqd 4
```

E. Annotating ancestral traits

Augur can use the time tree to infer the region and country of all internal nodes. The ancestral traits for all nodes can be annotated using the following command:

```
augur traits --tree <refined_ncov_tree.nwk> --metadata <metadata.tsv> --  
output <ncov_traits.json> --columns region country --confidence --sampling-  
bias-correction 2.5
```

For the geographic region-focused analysis, use the following command:

```
augur traits --tree <refined_ncov_tree_india.nwk> --metadata <metadata.tsv>  
--output <ncov_traits_india.json> --columns city --confidence --sampling-  
bias-correction 2.5
```

F. Inferring ancestral sequences and nucleotide mutations

The following command will identify the nucleotide mutations of the branches of the tree and infer the ancestral strain of each node:

```
augur      ancestral      --tree      <refined_ncov_tree.nwk>      --alignment
<aligned_ncov.fasta>  --output-node-data  <ncov_nt_muts.json>  --inference
joint --infer-ambiguous
```

For the geographic region-focused analysis, use the following command:

```
augur      ancestral      --tree      <refined_ncov_tree_india.nwk>      --alignment
<aligned_ncov_india.fasta>  --output-node-data  <ncov_nt_muts_india.json>  --
inference joint --infer-ambiguous
```

G. Inferring amino acid mutations

The following command will identify the amino acid mutations using the reference genome and ancestral sequences:

```
augur      translate    --tree      <refined_ncov_tree.nwk>      --ancestral-sequences
<ncov_nt_muts.json>      --reference-sequence      <MN908947.gb>      --output
<ncov_aa_muts.json>
```

For the geographic region-focused analysis, use the following command:

```
augur translate --tree <refined_ncov_tree_india.nwk> --ancestral-sequences
<ncov_nt_muts_india.json> --reference-sequence <MN908947.gb> --output
<ncov_aa_muts_india.json>
```

H. Identifying clades

The following command will label clades within the dataset using the nucleotide and amino acid mutations specified in the clades.tsv file:

```
augur clades --tree <refined_ncov_tree.nwk> --mutations <ncov_aa_muts.json>
<ncov_nt_muts.json>      --clades      <clades.tsv>      --output-node-data
<ncov_clades.json>
```

For the geographical region-focused analysis, use the following command:

```
augur      clades      --tree      <refined_ncov_tree_india.nwk>      --mutations
<ncov_aa_muts_india.json> <ncov_nt_muts_india.json> --clades <clades.tsv> -
--output-node-data <ncov_clades_india.json>
```

I. Exporting output files for visualization

The following command will export all output files generated in the previous steps of the analysis as a
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single JSON file to visualize the data using Nextstrain:

```
augur export v2 --tree <refined_ncov_tree.nwk> --metadata <metadata.tsv> --node-data <branch_lengths_ncov.json> <ncov_aa_muts.json> <ncov_nt_muts.json> <ncov_traits.json> <ncov_clades.json> --auspice-config auspice_config.json --lat-longs lat_longtsv --colors colors.tsv --output auspice/COVID_global.json
```

For the geographic region-focused analysis, use the following command:

```
augur export v2 --tree <refined_ncov_tree_india.nwk> --metadata <metadata.tsv> --node-data <branch_lengths_ncov_india.json> <ncov_aa_muts_india.json> <ncov_nt_muts_india.json> <ncov_traits_india.json> <ncov_clades_india.json> --auspice-config auspice_config.json --lat-longs lat_longtsv --colors colors.tsv --output auspice/COVID_india.json
```

J. Viewing the data

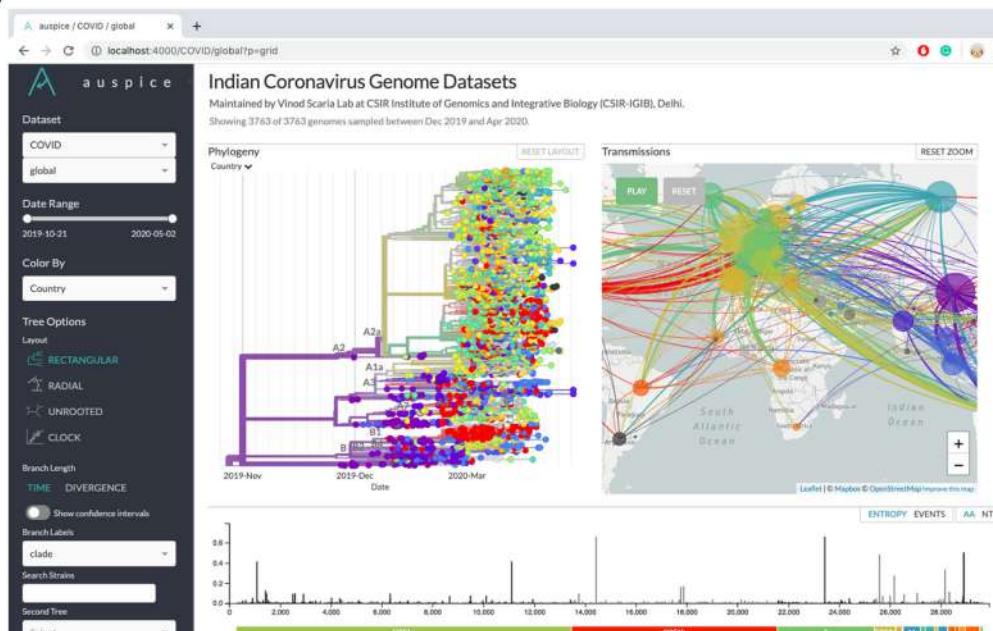
To visualize the output, use the following command:

```
nextstrain view auspice/ --allow-remote-access
```

This command will start the Auspice server on port 4000. The output can then be visualized through a browser by navigating to <http://127.0.0.1:4000/> or using the IP address of the machine on which the Auspice service is running and navigating to http://IP_ADDRESS_OF_MACHINE:4000/. The different subsampled datasets can be found under the ‘Dataset’ dropdown menu (Figure 6).

Note: For the links, the user will need to follow the steps given in the protocol. The hyperlinks correspond to a locally operated server through ‘Auspice’ (installation and instructions are detailed in the protocol), which helps the user to view the phylogeny on their own system through a browser.

(A)



(B)

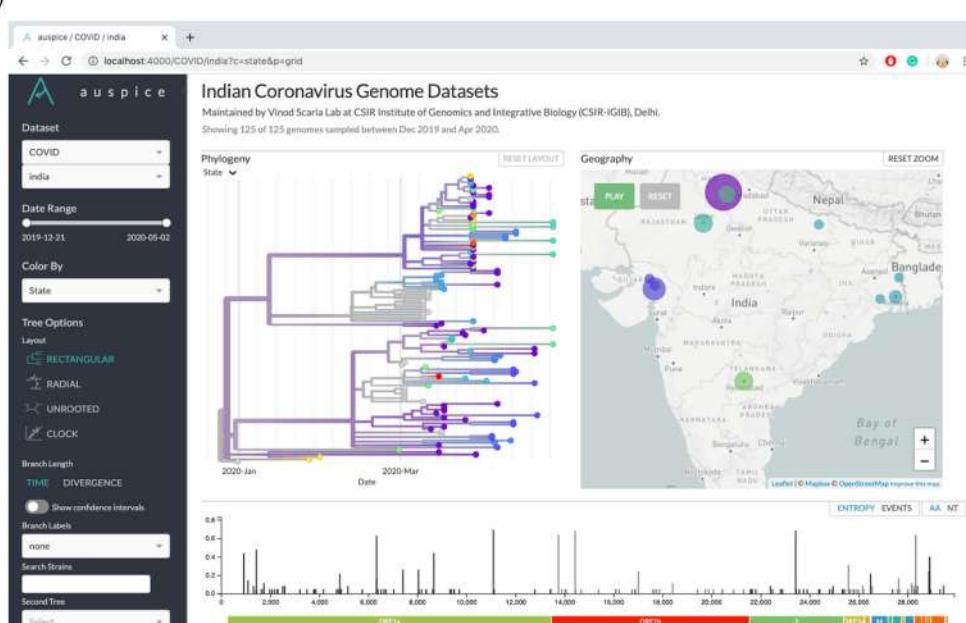


Figure 6. Screenshot of the visualization produced by Nextstrain for the COVID_global and COVID_india datasets

Acknowledgments

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Immunophenotyping and Intracellular Staining of Fixed Whole Blood for Mass Cytometry (CyTOF)

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[Abstract] In this report, we present the implementation of mass cytometry for intracellular staining using fixed whole blood. In our assay described here, 250 µl of whole blood, is stimulated *in vitro* with PMA/ionomycin (or left unstimulated), in the presence of secretion inhibitors (brefeldin A and monensin), lysed-fixed using SMART TUBE buffers, barcoded (optional), surface stained, fixed, stained for intracellular markers, fixed and DNA stained. Using 250 µl of whole blood from a healthy donor, we show that the expression of major lineage populations such as T cells, B cells, NK cells and monocytes, as well as cytokines such as CD4⁺ and CD8⁺ IFNγ and TNFα across multiple batches ($n = 27$) is consistent, with the co-efficient of variation (CVs) $\leq 21\%$, implying minimum inter-variability. For each major cell type, the percentage is reported as a percent of singlets. The percentage of cytokine expression in response to stimulation is reported as a percent of the immediate parent cell type. This protocol has a number of benefits: from a biological perspective, it can be applied to clinical studies especially where blood draw volumes are limiting. Technically, the protocol can be adapted for barcoding, which adds the benefits of more uniform sample staining as well as antibody conservation especially for large study cohorts. Finally, for studies involving infectious diseases including the current global COVID-19 pandemic, this protocol permits infectious samples to be fixed prior to processing and staining, thereby reducing biosafety risks.

Keywords: CyTOF, Mass cytometry, Flow cytometry, Barcoding, Fixed whole blood, Immune profiling

[Background] Non-invasive methods to obtain biological samples and single cell technologies are much sought after, as they provide an opportunity to comprehensively study human diseases. Blood not only provides for a minimally-invasive, cost effective and readily accessible source of immunological sample, but whole blood stimulation also serves as the closest mimic of the *in vivo* condition. Single-cell Mass Cytometry (or Cytometry by Time of Flight mass spectrometry, CyTOF) is ideally suited to broad profiling of the immune system, since it allows for > 40 parameter panels, with little to no spillover between channels, which is a significant advancement over the procedural limitations of fluorescence flow cytometry (Leipold *et al.*, 2015). Conversely, cell acquisition speed is significantly lower, and cell loss significantly higher, for CyTOF compared to fluorescence flow cytometry. In this report, we show the establishment of a reference panel of 39-anti human antibodies for mass cytometry that broadly identifies the major immune cell types, well established T and B cell subpopulations, activation markers, cytolytic markers and cytokines. To characterize these immune cell lineages and their functional states, the panel of anti-human heavy metal-conjugated monoclonal antibodies was selected to target the epitopes shown in Table 3, and to be

compatible with fixed whole blood samples (Fernandez and Maecker, 2015 and our unpublished data). The panel described here, comprises an almost equal number of pre-made and in-house conjugates. To build this panel, we implemented the recommended panel design guidelines for mass cytometry. Low-abundance targets were allocated to higher-sensitivity channels and antibodies were designated to channels to minimize potential spectral overlap (Takahashi *et al.*, 2017). All antibodies in the designed panel were then titrated to best discriminate the positive population from the negative. This panel can be further customized for specific hypothesis driven studies. In addition, by integrating barcoding (Behbehani *et al.*, 2014) within this framework, we also demonstrate the compatibility of this panel with large clinical studies, to minimize technical variability. The ability to freeze fixed blood samples is also convenient for assembly of sample sets for retrospective batched analysis, with decreased biosafety risks in the setting of infectious disease. With regard to SARS-CoV-2 or other infectious agents that might be present, whole blood fixation by the method described here has not been proven to be fully inactivating. But given the known effects of fixation on viral infectivity (Möller *et al.*, 2015), it provides an increased level of safety.

Materials and Reagents

1. Parafilm (PARAFILM, catalog number: H32207017002)
2. BD Vacutainer™ Plastic Blood Collection Tubes with Sodium Heparin: Conventional Stopper (Fisher Scientific, catalog number: 367874)
3. 96 Well, Square V-bottom deep well plate (Costar/Corning, catalog number: 3960)
4. Universal Lids for deep well plates (Corning, catalog number: 3099)
5. 5 ml polystyrene round-bottom tube with cell strainer cap (Falcon Coring, catalog number: 352235)
6. 1.8 ml cryotubes (NUNC, catalog number: 375418)
7. 50 ml polystyrene reservoir (Costar/Corning, catalog number: 4870)
8. 15 ml and 50 ml polypropylene conical tubes (Falcon, catalog numbers: 352096 and 352070, respectively)
9. 0.5 ml PCR tubes (Corning, catalog number: 3750)
10. 1.7 ml Microcentrifuge tubes (GeneMate, catalog number: C-3260-1)
11. 10 ml serological pipettes (Corning, catalog number: 4488)
12. 25 ml serological pipettes (Falcon, catalog number: 357525)
13. Freshly drawn whole blood in heparin green top tubes, from healthy donors or patients
14. EQ™ Four Element Calibration Beads (Fluidigm, catalog number: 201078)
15. Erythrocyte Lysis Buffer (Qiagen, catalog number: 79217)
16. Brefeldin A (Sigma-Aldrich, catalog number: B7651, stock concentration 5 mg/ml)
17. Monensin (Sigma-Aldrich, catalog number: M5273, stock concentration 5 mg/ml)
18. Phorbol 12-myriate 12 acetate (PMA) (Sigma-Aldrich, catalog number: P8139, stock concentration 1 mg/ml)
19. Ionomycin (Sigma-Aldrich, catalog number: I0634, stock concentration 1 mg/ml)
20. Stable Lyse-V2 (SMART TUBE Inc., catalog number: 3L7080)
21. Stable Store-V2 (SMART TUBE Inc., catalog number: 3S1988)



22. BD FACS™ Lysing Solution (BD, catalog number: 349202)
23. Cell-ID™ 20-Plex Pd Barcoding Kit (Fluidigm, catalog number: 201060)
24. 16% paraformaldehyde (PFA) (Alfa Aesar, catalog number: 43368)
25. Phenotypic and intracellular antibodies (filtered through 0.1 µm spin filters) (Millipore, catalog number: UFC30VV00)
26. 10x phosphate-buffered saline (PBS) (ROCKLAND, catalog number: MB-008)
27. MilliQ water

Note: Beakers or bottles used to store MilliQ water are not washed with soap, due to the barium content of most commercial soaps.
28. BSA (Sigma-Aldrich, catalog number: A7284)
29. 10% Sodium Azide (Teknova, catalog number: S0209)
30. 0.5 M EDTA (Gibco, catalog number: 15575-038)
31. Cell-ID™ Intercalator Ir (Fluidigm, catalog number: 201192A)
32. 10× saponin-based permeabilization buffer (eBioscience, catalog number: 00-8333-56)
33. CyPBS (see Recipes)
34. CyFACS buffer (see Recipes)

Equipment

1. -80°C freezer
2. Incubator at 37°C, 5% CO₂
3. Biosafety Cabinet (BSL-2 rated) or appropriate biosafety cabinet recommended for the pathogen being tested
4. Centrifuge (Eppendorf Centrifuge 5810R)
5. p2, p10, p20, p100, p200, p1000 single-channel calibrated micropipettes (Rainin)
6. p200 and p1200 multi-channel calibrated micropipettes (Rainin)
7. 12 pin aspirator (V&P Scientific, Inc., catalog number: VP 187PC-3S)

Procedure

A. Whole Blood Stimulation and Fixation

1. Use a 96-well deep well plate and label the wells with the unstimulated (US) and stimulated conditions (PMA/ionomycin). Keep PMA/ionomycin stimulated wells spatially separated (at least one well apart) from unstimulated or antigen-stimulated wells, to avoid cross-contamination and false positive signals.
2. Invert the heparin green top tube a few times to mix the freshly drawn whole blood.
3. Using a p1000 single-channel pipette, transfer 250 µl of whole blood (WB) into the labeled deep well plate.
4. Add secretion inhibitors Brefeldin A and Monensin to both unstimulated and stimulated conditions. Add PMA/ionomycin stimulants to the wells containing whole blood to be stimulated (Tables 1 and

- 2). Pipette up and down to mix well.

Table 1. Protein secretion inhibitors

Reagent	Stock concentration	Intermediate dilution	Final concentration in stimulation wells	in cell
Brefeldin A	5 mg/ml in DMSO (stored in aliquots at -20°C)	1:10 in CyPBS	5 µg/ml (1:100), 2.5 µl in 250 µl	
Monensin	5 mg/ml in ethanol (stored in aliquots at -20°C)	1:10 in CyPBS	5 µg/ml (1:100), 2.5 µl in 250 µl	

Table 2. Activators

Reagent	Stock concentration	Intermediate dilution	Final concentration in cell stimulation wells	in cell
Phorbol 12-myristate 13-acetate (PMA)	1 mg/ml in DMSO (stored in aliquots at -20°C)	1:1,000 in CyPBS	10 ng/ml, 2.5 µl in 250 µl	
Ionomycin	1 mg/ml in DMSO (stored in aliquots at -20°C)	1:10 in CyPBS	1 µg/ml, 2.5 µl in 250 µl	

5. Incubate the deep well plate for 4 h in an incubator at 37°C, 5% CO₂.
6. At the end of the incubation, add 350 µl Stable-Lyse V2 (at room temperature) per 250 µl whole blood. Add the Stable-Lyse V2 in the same order as the addition of the secretion inhibitors and stimuli to the whole blood to maintain a consistent incubation time. Pipette up and down to mix the contents of the well. Incubate at room temperature (RT) for 15 min.
7. At the end of the 15 min incubation, immediately add 1,000 µl Stable-Store V2 (at room temperature) per 250 µl whole blood. Add the Stable-Store V2 in the same order to the wells as maintained in Steps A4 and A6 to maintain a consistent incubation time. Pipette up and down to mix well. Incubate at RT for 15 min. Total volume per well will be 1.6 ml (250 µl WB + 350 µl stable-lyse V2 + 1,000 µl stable-store V2).
8. At the end of the incubation, transfer 1.6 ml of the unstimulated and stimulated fixed whole blood sample to cryo-labeled tubes and place in -80°C freezer until the samples are ready to be thawed and stained.

Note: Samples are transferred to cryovials for freezing, to facilitate quick sample-thaw. Depending on the study design, samples, may be frozen in the deep well plate.

B. Thaw Fixed Whole Blood Samples

1. Remove samples from -80°C freezer and thaw in cold water for about 15 min.
2. Transfer the WB samples (1.6 ml) to a deep well plate.
3. Centrifuge cells at 974 × g for 10 min at 4°C.
4. Aspirate the supernatant using the 8-12 pin aspirator.
5. (Optional step) If red blood cells (RBCs) are observed upon thawing the samples, add 1 ml of Erythrocyte Lysis Buffer (does not contain fixative). Incubate at RT up to a maximum of 10 min

(watch for lysis). Stop the lysis by adding 0.8 ml of CyFACS buffer and proceed to Step B7.

6. If no RBCs are observed, add 1 ml CyFACS buffer.
7. Centrifuge cells at $974 \times g$ for 10 min at 4°C .
8. Repeat wash with 1 ml CyFACS buffer.
9. Centrifuge cells at $974 \times g$ for 10 min at 4°C .

Note: Cell count/sample post 2 CyFACS washes should be $\sim 1 \times 10^6$ cells.

10. Cells are now ready to be barcoded or surface stained as required.

Note: If samples are not barcoded, skip Procedure C and proceed to Procedure D of surface staining.

C. Barcoding

1. Barcode Perm Buffer: Prepare 4 ml for each sample to barcode by mixing 1 part Maxpar 10 \times Barcode Perm with 9 parts Maxpar PBS; store at 4°C for up to one week.
2. Wash each sample with 1 ml Barcode Perm Buffer. Centrifuge cells at $974 \times g$ for 10 min. Aspirate supernatant from the cells.
3. Repeat washes 2 \times with 1 ml Barcode Perm Buffer.
4. Resuspend each sample to be barcoded completely in 800 μl Barcode Perm Buffer.
5. Resuspend each barcode tube containing 10 μl of the pre-mixed barcode completely in 100 μl Barcode Perm Buffer.
6. Transfer 110 μl of barcodes to the appropriate samples.
7. Mix the sample with the barcodes immediately and completely by pipetting.
8. Incubate the samples with the barcodes for 30 min at RT.
9. Wash cells thrice with 2 ml of Maxpar Cell Staining Buffer. Centrifuge cells at $974 \times g$ for 10 min. Aspirate supernatant from the cells.
10. Resuspend in 100 μl Maxpar Cell Staining Buffer.
11. Combine all barcoded samples into one well.
12. Centrifuge cells at $974 \times g$ for 10 min. Aspirate supernatant from the cells.

D. Surface staining

1. Make the surface antibody cocktail in CyFACS buffer of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Prepare sufficient volume of the antibody cocktail (Table 3) to add 200 μl of the cocktail for a barcoded pool of 10 samples or 70 μl of the antibody cocktail/ sample for non-barcoded samples.

Table 3. CyTOF panel for the phenotypic and functional analysis of immune cell subsets in fixed whole blood

Metal Label	Specificity	Ab. Clone	Source	Staining step
89 Y	CD66b	G10F5	In-house	Surface
102Pd-110Pd	Barcode	optional		
113Id	CD57	HCD57	In-house	Surface

140Ce	Beads	n/a		
141Pr	HLA-DR	L243 (G46-6)	In-house	Surface
142Nd	CD19	HIB19	Fluidigm	Surface
143Nd	IL-10	JES3-9D7	In-house	Intracellular
144Nd	1L-4	MP4-25D2	Fluidigm	Intracellular
145Nd	CD4	RPA-T4	Fluidigm	Surface
146Nd	CD8	RPA-T8	Fluidigm	Surface
147Sm	CD20	2H7	Fluidigm	Surface
148Nd	CD40	5C3	In-house	Surface
149Sm	CD11c	Bu15	In-house	Surface
150Nd	CD123	6H6	In-house	Surface
151Eu	CD107a	H4A3	Fluidigm	Intracellular
152Sm	TNF α	Mab11	Fluidigm	Intracellular
153Eu	CD45RA	HI100	Fluidigm	Surface
154Sm	CD3	UCHT1	Fluidigm	Surface
155Gd	CD45	HI30	In-house	Surface
156Gd	CD38	HB-7	In-house	Surface
157Gd	CD25	M-A251	In-house	Surface
158Gd	CD33	P67.6	In-house	Surface
159Tb	GM-CSF	BVD2-21C11	Fluidigm	Intracellular
160Gd	CD14	M5E2	Fluidigm	Surface
161Dy	IFN γ	4S.B3	In-house	Intracellular
162Dy	CD69	FN50	Fluidigm	Intracellular
163Dy	TCR $\gamma\delta$	B1	In-house	Surface
164Dy	IL-17	N49-653	Fluidigm	Intracellular
165Ho	CD127	A019D5	Fluidigm	Surface
166Er	IL-2	MQ1-17H12	Fluidigm	Surface
167Er	CD27	L128	Fluidigm	Surface
168Er	CD154 (CD40L)	24-31	Fluidigm	Intracellular
169Tm	CCR7	150503	In-house	Surface
170Er	PD1	EH12.1	In-house	Surface
171Yb	Granzyme B	GB11	Fluidigm	Intracellular
172Yb	FcRL5	509f6	In-house	Intracellular
173Yb	Perforin	B-D48	In-house	Intracellular
174Yb	CD21	Bu32	In-house	Surface
175Lu	IgD	IA6-2	In-house	Surface
176Yb	CD56	NCAM16.2	Fluidigm	Surface
191Ir	DNA1	n/a		
193Ir	DNA2	n/a		
209Bi	CD16	3G8	Fluidigm	Surface

2. Transfer the surface antibody cocktail into a 0.1 μ m spin filter and centrifuge using a tabletop microcentrifuge (RCF = 14,000 or max speed) for 2 min at room temperature.
3. For a barcoded sample pool (\times 10 conditions) add 200 μ l surface antibody cocktail.

4. Add 70 μ l surface antibody cocktail to each non-barcoded sample.
5. Incubate the cells at RT for 30 min.
6. Wash cells with 1 ml CyFACS buffer. Centrifuge cells at $974 \times g$ for 10 min. Discard supernatant by aspiration.
7. Repeat CyFACS wash. Centrifuge cells at $974 \times g$ for 10 min. Aspirate the supernatant.
8. Fix: Add 1.8 ml of BD FACS Lysing solution (stock diluted 1 \times with MilliQ water) to all samples.
9. Incubate cells overnight at 4°C.

E. Intracellular staining

1. Centrifuge cells at $974 \times g$ for 10 min at 4°C. Discard supernatant by aspiration.
2. Prepare 1 \times Perme (eioscience permeabilization) Buffer (1:10 dilution with MilliQ water). Add 1 ml Perm buffer.
3. Centrifuge cells at $974 \times g$ for 10 min at 4°C.
4. Repeat wash with Perm buffer.
5. Centrifuge cells at $974 \times g$ for 10 min at 4°C.
6. Make the intracellular antibody cocktail (Table 3) in 1 \times Perm buffer of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Prepare sufficient volume of the antibody cocktail to add 200 μ l of the cocktail for a barcoded sample pool of 10 samples and 70 μ l of the antibody cocktail/sample for non-barcoded samples.
7. Transfer the intracellular antibody cocktail into a 0.1 μ m spin filter and centrifuge using a tabletop microcentrifuge (RCF = 14,000 or max speed) for 2 min at room temperature.
8. For a barcoded sample pool (\times 10 conditions) add 200 μ l of intracellular antibody cocktail.
9. Add 70 μ l intracellular antibody cocktail to each non-barcoded sample.
10. Incubate the cells at RT for 30 min.
11. Wash cells with 1 ml CyFACS buffer. Centrifuge cells at $974 \times g$ for 10 min. Discard supernatant by aspiration.
12. Repeat 2 \times CyFACS washes. Centrifuge cells at $974 \times g$ for 10 min. Discard supernatant by aspiration.
13. Fix: Prepare 2% PFA from 16% PFA in CyPBS.
14. Add 200 μ l of 2% PFA in CyPBS to each sample.
15. Incubate at 4 °C overnight.

F. DNA staining

1. Add 1 ml CyFACS buffer to each sample.
2. Centrifuge cells at $974 \times g$ for 10 min at 4°C. Discard supernatant by aspiration.
3. Prepare 1:1,000 dilution in 2% PFA (in CyPBS) of Ir-intercalator.
4. Add 300 μ l of diluted Ir-intercalator to each sample and pipet to mix thoroughly.
5. Incubate at RT for 20 min.
6. Wash 2 \times in CyFACS buffer. Centrifuge cells at $974 \times g$ for 10 min at 4°C. Discard supernatant by aspiration.

7. Wash 2 × with MilliQ water. Centrifuge cells at $974 \times g$ for 10 min at 4°C . Discard supernatant by aspiration.
8. Using a cell strainer, dilute cells with EQ™ Four Element Calibration Beads (1:10 dilution with MilliQ water). Aim for a cell concentration of 0.6-1.0 million cells/ml.
9. Acquire samples (250k events per sample) on the CyTOF machine, after standard instrument set up.

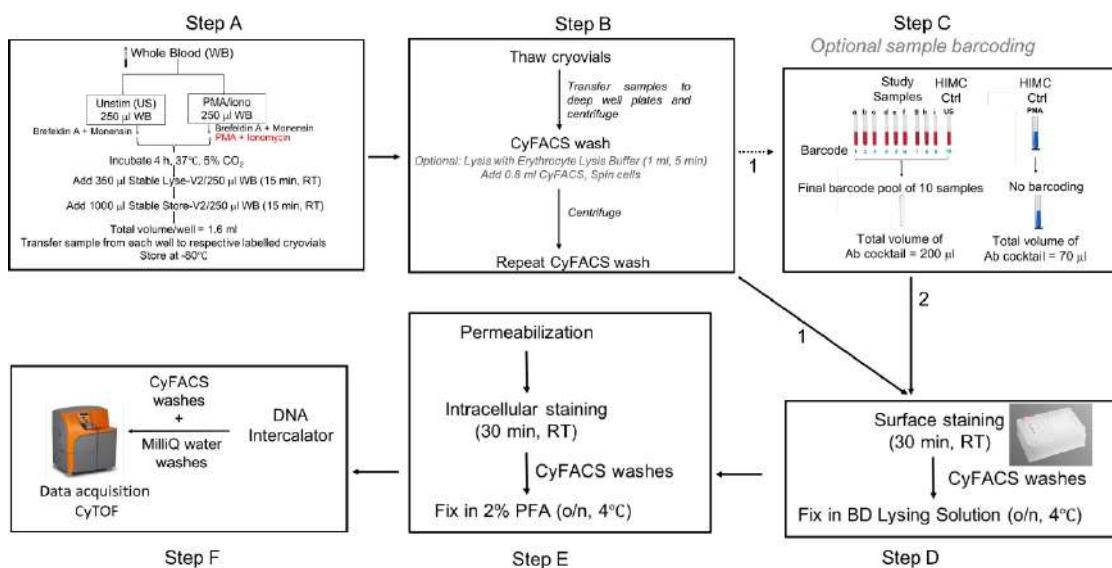


Figure 1. Schematic of the steps described in the procedure

Data analysis

Identification of Cell Lineages and Functional Subsets

After acquisition on the Helios instrument, FCS files are obtained for downstream analysis. If EQ beads (Fluidigm) are added, the files can be normalized based on EQ bead intensity, using the Nolan Lab MATLAB normalizer available freely on github.com (<https://github.com/nolanlab/bead-normalization/releases>). The normalized files can then be uploaded to Cytobank (www.cytobank.org) or analyzed in another software such as FlowJo (BD Biosciences) for manual gating. A gating schematic for both barcoded and non-barcoded samples (Figures 2A, 2B, respectively) is shown to demonstrate the application of the panel to both approaches. Hierarchical gating is performed using ¹⁹¹Ir and ¹⁹³Ir DNA intercalator, ¹⁴⁰Ce beads and the event length parameter to discern intact singlets from debris and cell aggregates. All other major immune cell populations are sequentially identified using the lineage surface protein markers as indicated in the respective lineage plots (Figures 2A and 2B). Our panel also enables the identification of immune cell subpopulations such as memory T subsets (CD45RA, CD27 on CD4⁺ and CD8⁺ T cells), Tregs (CD4⁺CD25^{hi}CD127^{low}), activation markers (HLA-DR and CD38 on CD4⁺ and CD8⁺ T cells), plasmablasts (CD27^{hi}CD38^{hi} on CD19⁺CD20⁺ B cells) and stages of isotype-switched naïve and memory B cells (CD27, IgD on CD19⁺CD20⁺ B cells) as indicated in the respective defined plots (Figures 2A and 2B). Figure 2C shows the cytokine expression of TNF α and IFN γ upon PMA/ionomycin stimulation on CD4⁺ and CD8⁺ T cells.

Data Reproducibility across Different Batches

To test the reproducibility of the panel, we analyzed data from replicate frozen vials of a fixed control blood sample, previously unstimulated or stimulated with PMA+ionomycin. These control sample replicates were stained and run in 27 separate batches. For each batch, we processed the unstimulated sample replicate in the context of 10 barcoded samples that included other donors and stimulations (Figure 2A), whereas the PMA+ionomycin stimulated replicate was stained and run independently in the same batch (without barcoding). This was to prevent false positive signals due to potential contamination of barcoded samples with the highly-stimulated PMA+ionomycin sample (Figure 2B). We calculated the frequency of the lineage populations using singlets as the parent population (Figure 3A) as well as frequency of TNF α and IFN γ for both CD4 $^{+}$ and CD8 $^{+}$ T cells (Figure 3C) (Leipold *et al.*, 2018). The percent co-efficient of variation (CV) was calculated to determine the degree of variability of expression across batches (Leipold *et al.*, 2018). Our analysis showed that the frequency of parent is comparable ($CV \leq 21\%$) across the 27 batches for both the lineage populations (Figure 3B) and cytokine expression (Figure 3D), suggesting minimum technical variability.

In summary, this study demonstrates the establishment and assessment of an ICS antibody panel for fixed whole blood that can be adapted to a barcoding approach.

Representative Data

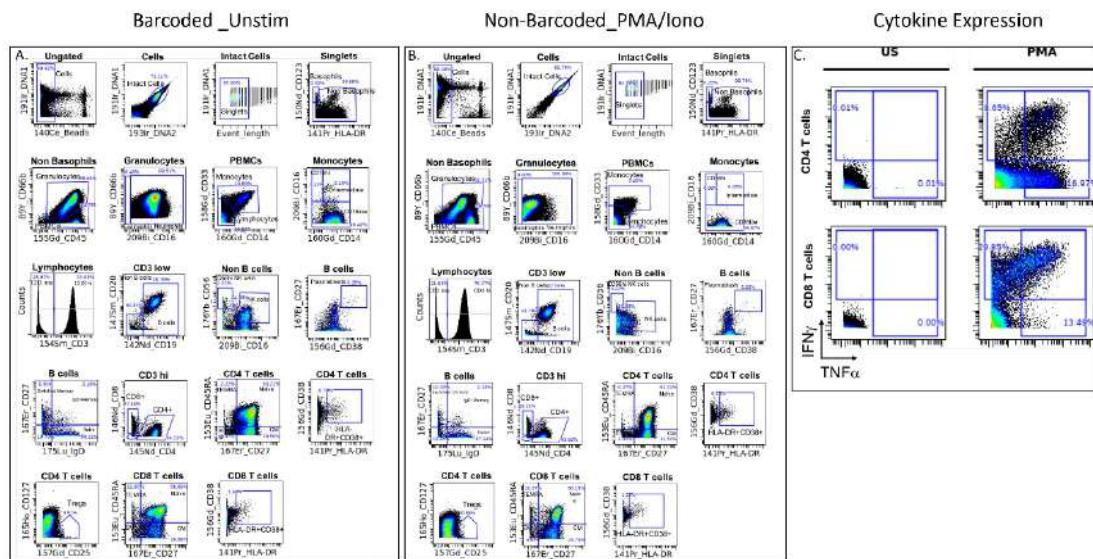


Figure 2. Schematic gating of fixed whole blood. The gating scheme is shown for fixed whole blood from a healthy donor processed in the same batch. The unstimulated condition (A) was included in a barcoded pool of 10 samples, while the PMA/ionomycin stimulated sample (B) was treated independently. Panel C shows the expression of two cytokines IFNy and TNFa for CD4 and CD8 T cells.

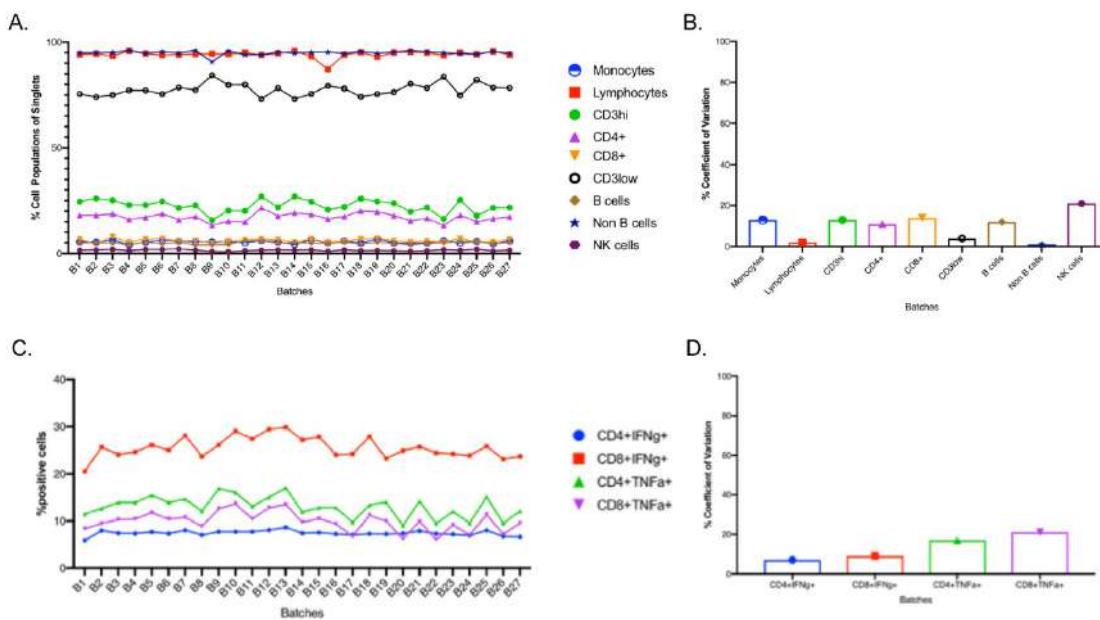


Figure 3. Expression of cell populations and cytokines across batches. A and C show the frequencies of cell lineages and functional subsets, respectively, across 27 batches of healthy control sample replicates. Frequencies are expressed as a percentage of singlets (3A) or of CD4 $^{+}$ or CD8 $^{+}$ T cells (3C). B and D show the percent coefficient of variation calculated across the 27 batches for the cell lineages (3B) and functional subsets (3D).

Recipes

1. CyPBS

1× PBS without heavy metal contaminants, such as 10× PBS

Made in MilliQ water

Sterile filter before use

2. CyFACS buffer

1× CyPBS with 0.1% BSA

2 mM EDTA (from 0.5 M EDTA stock)

0.05% sodium azide (from 10% stock)

Made in MilliQ water

Sterile filter before use

Acknowledgments

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Competing interests

The authors declare that they have no conflicts or competing interests.

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Production of the Receptor-binding Domain of the Viral Spike Proteins from 2003 and 2019 SARS CoVs and the Four Common Human Coronaviruses for Serologic Assays and Inhibitor Screening

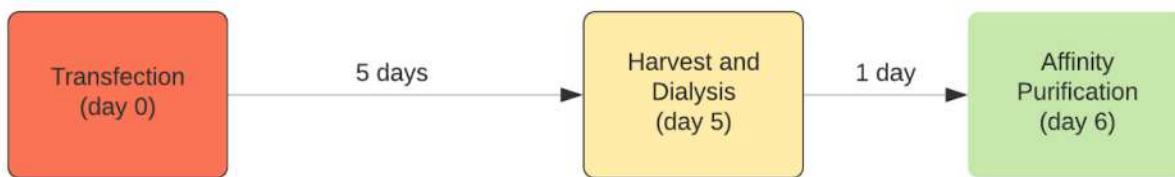
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[Abstract] The recombinant receptor-binding domain (RBD) of the viral spike protein from SARS-CoV-1 and 2 are reliable antigens for detecting viral-specific antibodies in humans. We and others have shown that the levels of RBD-binding antibodies and SARS-CoV-2 neutralizing antibodies in patients are correlated. Here, we report the expression and purification of properly folded RBD proteins from SARS and common-cold HCoVs in mammalian cells. RBD proteins were produced with cleavable tags for affinity purification from the cell culture medium and to support multiple immunoassay platforms and drug discovery efforts.

Graphic abstract:



High-Yield Production of Viral Spike RBDs for Diagnostics and Drug Discovery.

Keywords: SARS-CoV-2, SARS-CoV, Coronavirus, COVID-19, Antigen, Immunoassay, Halo-tag, Spike protein, Antibody, Inhibitor screening, Drug discovery

[Background] The receptor-binding domain (RBD) of the coronavirus spike protein is critical for viral attachment, fusion, and entry. It is also the primary target for antibody response and the development of entry inhibitors and vaccines. The RBDs of 2003 and 2019 SARS CoVs and the four common endemic human CoVs are poorly conserved, representing a promising antigen for detecting viral-specific antibodies in humans. We have recently shown that the RBD of SARS-CoV-2 is highly sensitive and specific for detecting antibodies nine days after the onset of symptoms (Premkumar et al., 2020). Levels of RBD-binding antibodies in human sera are strongly correlated with the SARS-CoV-2 neutralizing titer in patients. Thus, RBD-based serologic assays are attractive to identify individual and environmental risk factors for severe illness and to monitor SARS-CoV-2 transmission in the community. Prior immunity to common human endemic coronaviruses (229E, NL63, OC43, and HKU1) has been reported to enhance the inflammatory response to SARS-CoV-2 (Grifoni et al., 2020; Mateus et al., 2020). Here, we present a

detailed step-by-step method for expressing and purifying the RBD of 2003 and 2019 SAR CoVs and the four common endemic human CoVs for serologic assays and inhibitor screening (Premkumar et al., 2020 and Puhl et al., 2021). The technique allows the production of RBDs fused to a TEV protease cleavable self-labeling protein (HaloTag) at the N-terminus and a Twin-Strep-tag and a His-tag at the C-terminus. The tags were designed to aid affinity purification and oriented capture of antigens on solid supports incorporating streptactin, streptavidin, or nickel-nitrilotriacetic acid. The current protocol utilizes a mammalian expression system (Expi293) to produce milligram quantities of recombinant RBDs from a small cell culture volume within 5-7 days, using a single affinity purification step.

Materials and Reagents

1. Cryopreservation Tubes (Thermo Scientific, catalog number: 374081)
2. Poly-Prep Chromatography Columns (Bio-Rad, catalog number: 7311550)
3. Econo-Pac Chromatography Columns (Bio-Rad, catalog number: 7321010)
4. SnakeSkin Dialysis Tubing (Thermo Fisher Scientific, catalog number: 68700)
5. SnakeSkin Dialysis Clips (Thermo Fisher Scientific, catalog number: 68011)
6. Staples 2" Binder Clips, Large (Staples, catalog number: 10669)
7. 5 ml Serological pipette (Thermo Fisher Scientific, catalog number: 13-678-11D)
8. 10 ml Serological pipettes (Thermo Fisher Scientific, catalog number: 13-678-11E)
9. 50 ml Falcon tube (Cell star, catalog number: 22761)
10. Precision Plus Protein Kaleidoscope 500 ml (Bio-Rad, catalog number: 161-0375)
11. Any kD Mini-PROTEAN TGX Stain-Free Protein Gels, 12-well, 20 µl (Bio-Rad, catalog number: 4568125)
12. 2× Laemmli Sample Buffer (Bio-Rad, catalog number: 1610737)
13. Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels, 2-gel (Bio-Rad, catalog number: 1658005)
14. 2-Mercaptoethanol, 10 ml (Sigma-Aldrich, catalog number: M6250)
15. Coomassie Brilliant Blue R-250 Dye (Thermo Fisher Scientific, catalog number: 20278)
16. Expi293 Expression System Kit (Thermo Fisher Scientific, catalog number: A14635)

Note: Store cells in liquid nitrogen and other reagents at 2°C to 8°C.

17. Mr. Frosty (Thermo Scientific, catalog number: 5100-0001)
18. Ni-NTA Agarose (QIAGEN, catalog number: 30230). Store at 2°C to 8°C
19. DMSO (Millipore Sigma, catalog number: 41640)
20. Opti-MEM Medium (Thermo Fisher Scientific, catalog number: 11-058-021)
21. Tris (MP, catalog number: 103133)
22. NaCl (Fisher, catalog number: S271-10)
23. Glycerol (VWR, catalog number: BHD 1172-1LP)
24. Sucrose (Fisher, catalog number: BP-220-1)
25. Imidazole (Thermo Fisher Scientific, catalog number: 03196-500)
26. Liquid nitrogen (Arc gases)

27. Purify Buffer (see Recipes)
28. Elution Buffer (see Recipes)
29. Dialysis Buffer (see Recipes)
30. Stain Buffer for SDS PAGE (see Recipes)
31. Destain Buffer for SDS PAGE (see Recipes)

Equipment

1. Fisherbrand Shaker Flasks, Plain Bottom, Vented (Thermo Fisher Scientific, catalog number: PBV12-5). Store at room temperature
2. 4 L Beaker (Thermo Fisher Scientific, catalog number: 02-555-25K)
3. Forma Steri-Cycle i160 CO₂ Incubator (Thermo Fisher Scientific, Forma, catalog number: 51030301)
4. CO₂ Resistant Shaker (Thermo Fisher Scientific, catalog number: 88881101)
5. Biological safety cabinet (Labguard, Class II, Type A2)
6. Precision Water Bath GP 15D–5 L and 10 L (Thermo Scientific, catalog number: TSGP15D)
7. Magnetic stir bar (Thermo Fisher Scientific, catalog number: 14-512-136)
8. Centrifuge (Sorvall, model: RC-5B)
9. Centrifuge (Eppendorf, model: 5810 R)
10. Centrifuge (Thermo, model: Sorvall Legend Micro 21R)
11. Freezer (Thermo Scientific Revco RLE60086A -86°C)
12. Cryogenic dewar (Cole Parmer)
13. Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (Bio-Rad, Catalogue number: 1658004)

Procedure

- A. Establishment of the Expi293 Cell Line (Thermo Fisher Scientific)
 1. Remove a cell aliquot from liquid nitrogen.
 2. Immediately hand thaw the cells and place them in a 37°C water bath. Once thawed, swirl the tube gently without submerging completely until only a small amount of ice remains.
 3. Spray hands with 70% ethanol and gently rub the cell vial to decontaminate before transferring into the laminar flow hood.
 4. Use a serological pipette to transfer all tube contents into a plain bottom, vented Fisherbrand Shaker Flask, prewarmed with 30 ml of Expi293 Expression Medium.
 5. Incubate cells at 37°C with ≥ 80% relative humidity and 8% CO₂. Set shaking speed to 125 RPM for a 125 ml shaker flask.
 6. Passage cells when the cell density reaches 1×10^6 - 3×10^6 cell/ml.
Note: This usually occurs 4-6 days post-thaw.
 7. Proceed to transfection once the cell density reaches approximately 3×10^6 - 5×10^6 viable cells/ml and cell viability is ≥ 95%.

Note: To cryopreserve cells for future use, grow the cell culture to 3×10^6 - 5×10^6 viable cells/ml and centrifuge them at $300 \times g$ for 5 min.

- a. Discard the supernatant, add Expi293 Expression medium with 10% DMSO, and gently resuspend the cells by pipetting.
- b. Dilute the cells to 1×10^7 viable cells/ml and pipet 1 ml aliquots into cryopreservation tubes and freeze with a controlled-rate freezing apparatus at -80°C freezer. After 24 h, transfer to cryogenic dewar for long-term storage and future use. Allow cells to recover in culture for two more passages post-thaw before transfecting.

Note: For general cell maintenance, passage cells at 0.5×10^6 cell/ml when they reach a density of 3×10^6 - 5×10^6 cell/ml. Growing past 5×10^6 cell/ml is not recommended.

B. Transfection (30 ml)

1. Dilute a total of 75×10^6 cells to a final density of 3×10^6 cell/ml with 25 ml of prewarmed Expi293 Expression Medium in a 125 ml shaker flask.
2. Dilute 25 µg of RBD expression plasmid DNA in 1.5 ml of Opti-MEM Medium.
3. Dilute 80 µl of ExpiFectamine 293 Reagent in 1.4 ml of Opti-MEM Medium. Incubate the solution at room temperature for 5 min.
4. Add diluted plasmid DNA to the solution containing ExpiFectamine 293 Reagent and incubate at room temperature 10-20 min. The volume should be approximately 3 ml.
5. Transfer 3 ml of the solution into a shaker flask and incubate cells at 37°C with ≥ 80% relative humidity and 8% CO₂.
6. Eighteen to twenty-two hours post-transfection, add 150 µl of ExpiFectamine 293 Transfection Enhancer 1 and 1.5 ml of ExpiFectamine 293 Enhancer 2 to the shaker flask. Incubate cells for up to 5 days post-transfection.

Note: The procedure can be scaled up proportionally for larger transfections. Cell viability should be above 50% on day 5.

C. Harvest and Dialysis

1. Transfer the cell culture to a 50 ml Falcon tube and centrifuge at 3,000-5,000 × g for 5 min at 25°C (Figure 1).

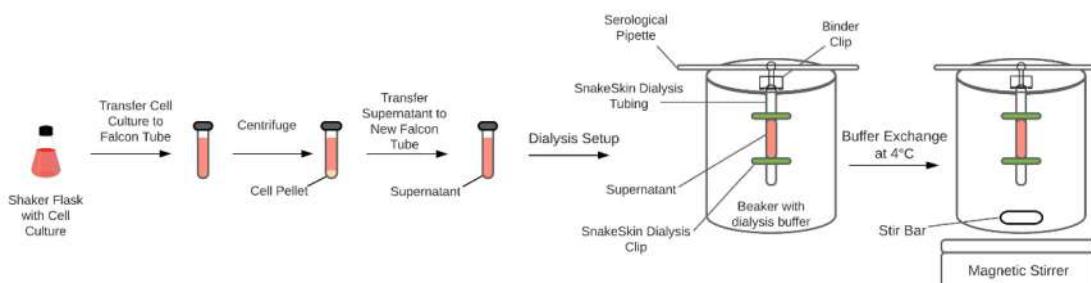


Figure 1. Schematics of harvesting cell culture and dialysis of supernatant

2. Transfer the supernatant into a fresh 50 ml Falcon tube and keep it on ice.
3. Prepare 3 L of dialysis buffer.
4. Use about 7-8 inches of SnakeSkin Dialysis Tubing and SnakeSkin Dialysis Clips to transfer 30 ml of harvested supernatant into the dialysis buffer.

Note: Hydrate the membrane with the dialysis buffer before transferring the supernatant.

5. Place the sealed snakeskin tubing and a magnetic stir bar in a 4-L beaker.
6. Place the beaker on a magnetic stirrer and allow buffer exchange at 4°C overnight.
7. Transfer the buffer-exchanged supernatant into a 50 ml Falcon tube.

Note: Upon harvesting, it is possible to evaluate the success of transfection and protein expression before the affinity chromatography step by SDS-PAGE with the cell culture supernatant (optional). To perform this:

- a. Mix 50 µl of 2× Laemmli Sample Buffer with 2-mercaptoethanol and 50 µl of cell culture supernatant and boil the sample at 95°C for 5 min.
- b. Load 8-15 µl of the reduced sample onto the SDS-PAGE.
- c. Run sample at 170 v in the Mini-PROTEAN Tetra Vertical Electrophoresis Cell for 35 min and visualize the bands after Coomassie staining using the manufacturer's protocol.

D. Immobilized metal affinity chromatography

1. Take 0.5 ml of Ni-NTA resin in a poly-prep chromatography column and equilibrate with 5 ml of purifying buffer in 1 ml increments. Close the column and resuspend the resin in 1 ml of purifying buffer.

Note: Proportionally adjust the amount of resin needed for larger transfections.

2. Transfer the equilibrated resin into a 50 ml Falcon tube containing the buffer exchanged supernatant.
3. Incubate the resin with the supernatant on a rocking shaker for 1 h at 4°C.
4. Remove the 50 ml Falcon tube from the rocker and place it on a stand. Allow the resin to settle for 20 min.
5. Transfer the supernatant into a new 50 ml Falcon tube without disturbing the resin.
6. Transfer the resin directly onto the bed of the poly-prep chromatography column.
7. Wash the resin with 6 ml of purifying buffer in 1 ml increments by pipetting on the chromatography column wall.

Note: Allow the buffer to flow through the column completely before adding another ml of wash buffer.

8. Elute the protein by adding 200 µl of elution buffer onto the column wall for a total of 7 fractions.
9. Quantify the protein by measuring the absorbance of the fraction at 280 nm.
10. Assess the protein purity by SDS PAGE run under reducing conditions (Figure 2).

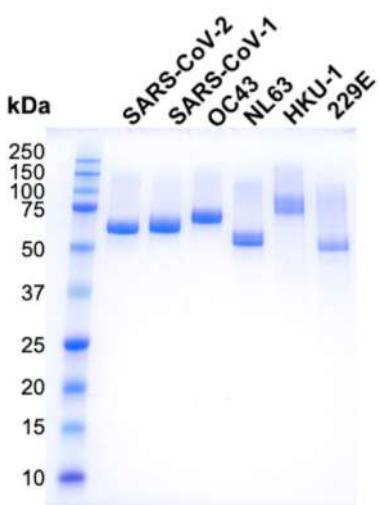


Figure 2. SDS-PAGE analysis of purified spike RBD proteins

E. Storage

Purified proteins can be stored at 4°C for a few weeks. For long-term storage, protein samples can be aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C. Frozen protein samples can be quickly hand thawed before use.

Recipes

1. Purify buffer

50 mM Tris pH 8

105 mM NaCl

10% glycerol

10% sucrose

2. Dialysis buffer

50 mM Tris-HCl, pH 8

100 mM NaCl

3. Elution buffer

50 mM Tris pH 8

105 mM NaCl

10% glycerol

10% sucrose

300 mM imidazole

4. Stain Buffer for SDS PAGE (1 L)

500 ml deionized water

100 ml methanol

100 ml glacial acetic acid

3 g brilliant blue

5. Destain Buffer for SDS PAGE (1 L)
500 ml deionized water
400 ml methanol
100 ml glacial acetic acid

Notes

Codon-optimized nucleotide sequences encoding the RBDs of SARS-CoV-1 (318-514 aa, P59594), SARS-CoV-2 (331-528 aa, QIS60558.1), OC43 (329-613 aa, P36334.1), HKU-1 (310-611 aa, Q0ZME7.1), 229E (295-433 aa, P15423.1), and NL63 (480-617 aa, Q6Q1S2.1) are available in GenBank under the accession codes MT649401, MT649402, MT649403, MT649404, MT649405, and MT649406. The genes encoding the proteins above were cloned between KpnI and Xhol sites of the mammalian expression plasmid p4H. The mammalian expression plasmids will also be made available by the authors from the plasmid repository (Addgene).

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Competing interests

The authors have declared no competing interest.

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Heterologous Expression and Purification of SARS-CoV2 Nucleocapsid Protein

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[Abstract] This protocol describes a step by step method for heterologous expression of SARS-CoV2 Nucleocapsid (N) protein in *Escherichia coli*. Moreover, this protocol includes steps to purify the N protein to high purity and homogeneity. Thus, purified protein can be used for ligand binding assays and other biochemical experiments.

Keywords: COVID-19, SARS CoV2, Nucleocapsid protein, Recombinant expression, Protein purification

[Background] Since its detection in late 2019 in Wuhan, China, SARS-CoV2 infections have been rampant around the world (Wu et al., 2020). In an effort to follow the course of early infections, the nucleocapsid protein (N) was used along with the spike protein (S) in serological assays to monitor the course of early infections in the New York area. N is one of the most highly expressed viral protein, and therefore a good target to follow. To facilitate these assays, a purification protocol was developed for N and used successfully in these studies.

Materials and Reagents

1. Syringe (VWR International, catalog number: BD309653)
2. 0.2 µm Syringe filters (VWR International, catalog number: 28196-368)
3. Econo-Column 2.5 × 10 cm (Bio-Rad, catalog number: 737-4251)
4. HiTrap Heparin HP column 5 ml (GE Healthcare, catalog number: 17040703)
5. Amicon Ultra-15 Centrifugal Filter Units 30 kD MWCO (Millipore, catalog number: UFC803096)
6. HiLoad 16/600 Superdex200 pg column (GE Healthcare, catalog number: 17106901)
7. 96-well Uniplate (Fisher Scientific, catalog number: 09-003-36)
8. Culture plates (Thermo Fisher Scientific, catalog number: 0 8-757-100D)
9. Culture flasks (Thermo Fisher Scientific, catalog number: 09-552-70)
10. *E. coli* Rosetta 2 (DE3) chemically competent cells (Novagen, catalog number: 71400-3)
11. pET28a(+)_Nucleocapsid plasmid [pET28a(+) vector (Novagen, catalog number: 69864-3) with SARS CoV2 Nucleocapsid encoding gene fused to an in frame C-terminal AALE linker and a 6xHis tag]

Note: pET28a(+)_nucleocapsid plasmid is available upon request from the corresponding author.

12. Agar (Fisher Scientific, catalog number: BP1423-500)

13. Yeast extract (Fisher Scientific, catalog number: BP1422-2)
14. Tryptone (Fisher Scientific, catalog number: BP1421-2)
15. Di-potassium hydrogen phosphate (K_2HPO_4) (Sigma-Aldrich, catalog number: 60353)
16. Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, catalog number: 92214)
17. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S8045)
18. 1,000× Kanamycin stock solution (50 mg/ml) (Gold Biotechnology, catalog number: K-120-25)
19. 1,000× Chloramphenicol stock solution (34 mg/ml) (Gold Biotechnology, catalog number: C-105-5)
20. 1 M IPTG (IsoPropyl-1-Thio-B-D-Galactopyranoside) (Gold Biotechnology, catalog number: I2481C50)
21. Tris (Gold Biotechnology, catalog number: T-095-1)
22. Sodium Chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
23. β -Mercaptoethanol (β -Me) (Sigma-Aldrich, catalog number: M6250-250ML)
24. 100% Glycerol (Fisher Scientific, catalog number: G33-4)
25. TurboNuclease (Accelagen, catalog number: N0103M)
26. Protease inhibitor cocktail (2 μ M Pepstatin, 6 μ M Leupeptin, 1 μ M PMSF, and 2 mM Benzamidine) (Sigma-Aldrich, catalog numbers: 11524488001, 11017128001, p7626-25, B6506-25)
27. Polyethylenimine (PEI) (Acros Organics, catalog number: AC178571000)
28. Ni-NTA Agarose resin (Qiagen, catalog number: 30250)
29. Bradford assay dye reagent concentrate (Bio-Rad, catalog number: 500-0006)
30. Imidazole (Affymetrix, catalog number: 17525)
31. ATP (P212121, catalog number: CI-00015-25G)
32. 12% Mini-PROTEAN TGX Precast protein gel, 15-well (Bio-Rad, catalog number: 4561046)
33. 4× Laemmli protein sample buffer for SDS-PAGE (Bio-Rad, catalog number: 1610747)
34. InstantBlue Ultrafast protein gel stain (VWR Scientific, catalog number: 95045-070)
35. Liquid Nitrogen
36. Luria-Bertani (LB) medium (see Recipes)
37. Terrific Broth (TB) medium (see Recipes)
38. Resuspension buffer (see Recipes)
39. Ni-NTA buffers
 - Wash buffer (see Recipes)
 - Elution buffer 1 (see Recipes)
 - Elution buffer 2 (see Recipes)
40. Heparin buffers
 - Heparin buffer A (see Recipes)
 - Heparin buffer B (see Recipes)
 - Heparin dilution buffer (see Recipes)
41. Size exclusion (SEC) buffer (see Recipes)
42. 10× SDS-PAGE running buffer (see Recipes)

Equipment

1. Nalgene PPCO Centrifuge bottles (1 L) (Fisher Scientific, catalog number: 05-562-25)
2. Ultracentrifuge tubes (Polycarbonate bottle, 38 × 102 mm, 70 ml) (Beckman Coulter Life Sciences, catalog number: 355655)
3. Beaker (Sigma-Aldrich)
4. Magnetic stirrer (Sigma-Aldrich)
5. Rocker (IBI Scientific)
6. Sonicator (12.7 mm tip) (QSonica LLC)
7. Incubator (at 37°C)
8. Incubator shaker (Eppendorf New Brunswick Innova)
9. Centrifuge (Beckman Coulter Life Sciences, model: J6-MI)
10. Rotor (Beckman Coulter Life Sciences, model: JS 4.3)
11. Ultracentrifuge (Beckman Coulter Life Sciences)
12. Ultracentrifuge Rotor (Beckman Coulter Life Sciences, model: Ti45)
13. Mini-PROTEAN® Tetra Vertical Electrophoresis Cell system (Bio-Rad, catalog number: 165-8005)
14. AKTA pure FPLC system (GE Healthcare)
15. Cold room
16. UV-VIS spectrophotometer (Thermo Fisher Scientific, model: NanoDrop1000)

Procedure

1. SARS CoV2 N-protein was cloned into pET28a(+) with an in-frame AAALÉ linker and 6xHis tag at C-terminal.
2. Transform 50 µl of chemically competent *E. coli* Rosetta 2 (DE3) cells with the 50-100 ng pET28a(+)_Nucleocapsid plasmid according to manufactures instructions. Plate the revival mix onto LB agar plate with Kanamycin (Kan) (50 µg/ml) and Chloramphenicol (Cam) (34 µg/ml) antibiotics, and leave it for incubation overnight in 37°C incubator.
3. Select a single colony from the LB agar plate and inoculate into 100 ml LB media (supplemented with Kan [50 µg/ml] and Cam [34 µg/ml]) at 37°C at 220 rpm shaking.
4. Inoculate 5-10 L TB media (supplemented with 50 µg/ml Kan and 34 µg/ml Cam) with 1% overnight culture and shake at 200 rpm at 37°C till the cells grow to an OD of 1.5.
5. Induce protein expression by adding IPTG to final concentration of 0.5 mM, and reduce the culture temperature to 18°C. Let the culture shake at 180 rpm for next 16-18 h.
6. Harvest the cells by centrifuging culture at 4,000 × g for 20 min at 4°C, followed by resuspending the pellet in 20 ml resuspension buffer per liter culture. Pellet can be stored frozen in -80°C for long term storage. Further purification steps were performed with samples either on ice or in cold room. Where otherwise, temperature is mentioned.

7. Thaw out the pellet in water bath (at 30°C) and add 2 U/ml TurboNuclease in the lysate, and lyse the cells by sonication at 100% amplitude for 4-5 min (3 s pulse on and 6 s pulse off). For large volumes (> 150 ml), divide the lysate into two beakers and sonicate separately.
8. Add PEI (final 0.2%) to the lysate while mixing with a magnetic stirrer for 5 min and ultracentrifuge the lysate for 1 h at 95,000 x g.
9. Filter the cleared lysate through a 0.2 µm syringe filter. Equilibrate the Ni-NTA agarose beads (0.5 ml beads per liter culture) in Wash buffer and add it to the cleared lysate. Let the Ni-NTA beads incubate on a rocker for 1 h in cold room. [Total volume of Ni-NTA beads = 1 column volume (CV)]
10. Separate the Ni-NTA beads from the lysate under gravity flow using Econo-column.
11. Wash the beads with 20 column volumes (CV) of Wash buffer and then elute the protein in Elution buffer 1 (4 fractions of 1 CV each). Most of the protein elutes out from the Ni-NTA beads with maximum protein in fraction 3 (Figure 1).
12. Elute the remaining protein with Elution buffer 2 (2 fractions of 2 CV each).
13. Measure the protein amount with Bradford method according to manufacturer's instructions.
14. Dilute the 10 ul of collected fractions with the 4x Laemmli protein sample buffer, heat for 5 min at 95°C and resolve the samples on SDS-PAGE at 200 V to analyze the purity of protein (Figure 1).

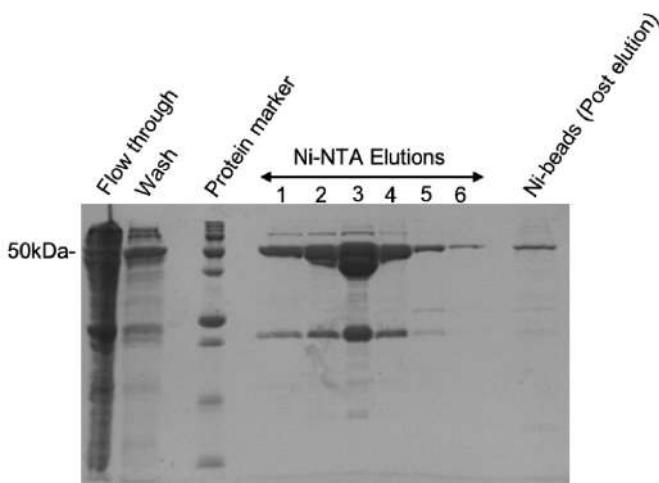


Figure 1. A representative SDS-PAGE for Ni-NTA affinity chromatography for SARS CoV2 N-protein. Different samples collected during Ni-NTA were analyzed on 12% SDS-PAGE. Elution fractions are numbered on top of the gel. Fractions 1-4 and 5-6 show protein eluted with Elution buffers 1 and 2 respectively. Some protein stays bound to Ni-beads after elution with 500 mM imidazole buffer.

15. Pool the elution fractions and dilute 1:1 using Heparin dilution buffer to reduce the NaCl concentration to 100 mM.
16. Connect HiTrap Heparin-HP column to AKTA pure system and equilibrate the column with 5 CV Heparin buffer A before loading the diluted protein on column using the sample pump. A constant flow rate of 4 ml/min was used for Heparin chromatography.

17. Wash the Heparin HP column with 8 CV Heparin buffer A, followed by protein elution using a linear gradient from 10 to 100% Heparin buffer B over 150 ml. Protein will elute between 60-70% Heparin buffer B concentration.
18. Collect 1-2 ml fractions in clean tubes and run the peak fractions on SDS-PAGE for analyzing the purity of the protein. A typical Heparin-HP elution chromatogram for N-protein is shown in Figure 2.

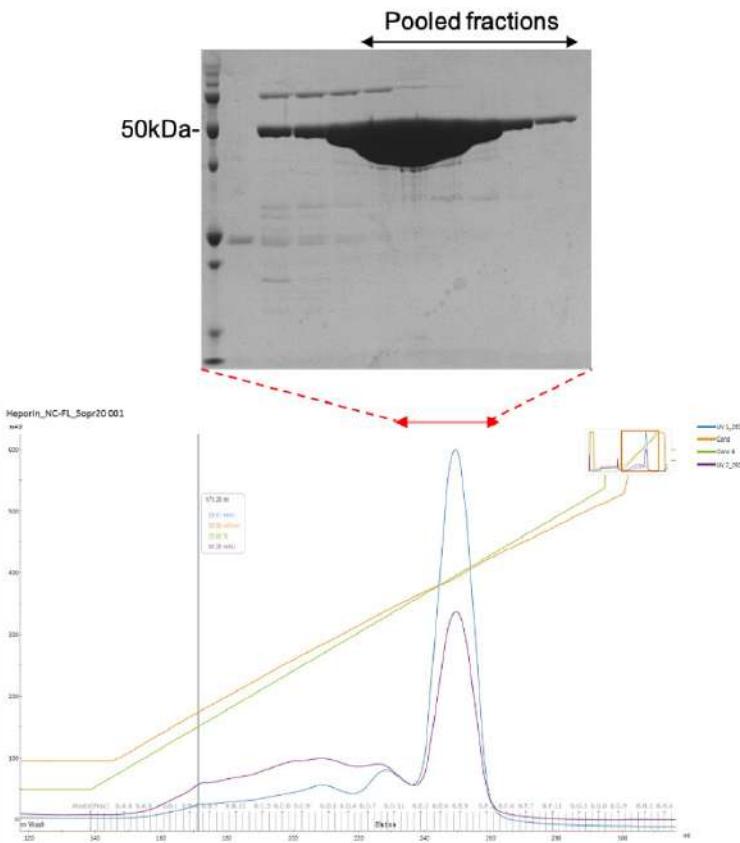


Figure 2. A representative SDS-PAGE and chromatogram for Heparin HP (5 ml) chromatography for SARS CoV2 N-protein. N-proteins eluted out between 60-70% Heparin buffer B and the fractions covering whole peak (marked with red arrow on top of the peak) were analyzed on 12% SDS-PAGE. Fractions having pure protein were further purified on size exclusion chromatography.

19. Pool the pure fractions from HiTrap Heparin HP elutions and concentrate it to ~2 ml final volume using 30 kD MWCO Amicon Ultra-15 Centrifugal Filter Units as per manufacturer's instructions.
20. Equilibrate the HiLoad 16/600 Superdex200 pg column with SEC buffer and inject the concentrated protein using capillary loop followed by eluting it out with SEC buffer at 1 ml/min flow rate. A single peak for the homogenous protein will be observed.
21. Collect 1 ml fractions in clean tubes and run the peak fractions on SDS-PAGE for analyzing the protein purity (Figure 3).

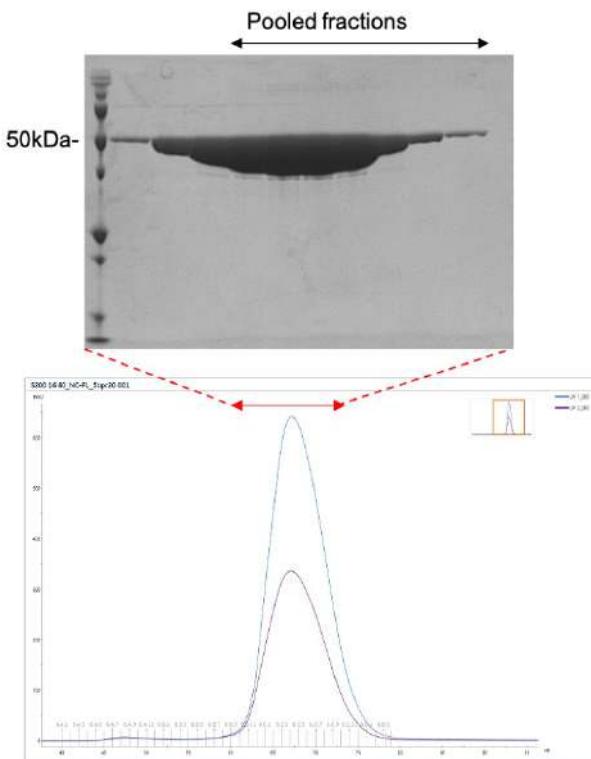


Figure 3. A representative SDS-PAGE and chromatogram for the size exclusion chromatography for SARS CoV2 N-protein. Concentrated protein was loaded on HiLoad 16/600 Superdex200 pg column and fractions covering whole peak (marked with red arrow in chromatogram) were analyzed on a 12% SDS-PAGE. Only pure protein containing fractions (marked on top of the gel) were pooled, concentrated and stored.

22. Pool the pure protein containing fractions and concentrate using 30 kD MWCO Amicon Ultra-15 Centrifugal Filter Units to desired concentration.
23. Protein aliquot can be flash frozen in liquid nitrogen and stored at -80°C for long term storage.

Data analysis

1. This section explains how to determine the yield and purity of protein after size exclusion chromatography.
 - a. A Spectrophotometer/NanoDrop can be used to measure the absorbance at 280 nm and 260 nm.
 - b. The 280 nm absorbance and the correction factor of 0.932 was used to determine the real concentration of N protein (Real cons = $A_{280}/0.932$). Purified protein shows a 260/280 ratio between 0.55-0.60, which represents that there is no nucleic acid contamination in the protein.
2. The UNICORN 7.4 software (GE Healthcare) was used to visualize the chromatograms and preparing chromatogram figures.

Recipes

1. LB medium
 - a. Weigh out 10 g tryptone, 5 g yeast extract, and 10 g NaCl
 - b. Add up to 900 ml deionized water
 - c. Adjust pH to 7.5 with 5 N NaOH
 - d. Adjust the final volume to 1 L with deionized water
 - e. Sterilize by autoclaving at 121 °C for 30 min
 - f. Store at room temperature
2. TB medium
 - a. Add 900 ml water to 20 g tryptone, 24 g yeast extract and 4 ml glycerol
 - b. Stir until the solutes have dissolved and sterilize by autoclaving
 - c. Prepare 100 ml Phosphate buffer (0.17 M KH₂PO₄ + 0.72 M K₂HPO₄) and sterilize by autoclaving
 - d. When at room temperature mix both the solutions and use immediately
3. Resuspension buffer

Tris	25 mM (pH 8.0)
NaCl	500 mM
β-Me	2 mM
Glycerol	5%
Imidazole	10 mM
Protease inhibitor cocktail	
4. Ni-NTA buffers
 - a. Wash buffer

Tris	25 mM (pH 7.4)
NaCl	500 mM
β-Me	2 mM
Glycerol	5%
Imidazole	50 mM
ATP	1 mM
 - b. Elution buffer 1

Tris	25 mM (pH 7.4)
NaCl	200 mM
β-Me	2 mM
Glycerol	5%
Imidazole	250 mM
 - c. Elution buffer 2

Tris	25 mM (pH 7.4)
NaCl	200 mM
β-Me	2 mM

Glycerol	5%
Imidazole	500 mM

5. Heparin buffers

a. Heparin buffer A

Tris	25 mM (pH 7.4)
NaCl	100 mM
β-Me	2 mM

b. Heparin buffer B

Tris	25 mM (pH 7.4)
NaCl	1 M
β-Me	2 mM

c. Heparin dilution buffer

Tris	25 mM (pH 7.4)
β-Me	2 mM

6. Size exclusion (SEC) buffer

Tris	25 mM (pH 8.0)
NaCl	500 mM
β-Me	2 mM

7. 10x SDS-PAGE running buffer

- Dissolve 30 g Tris base, 144 g glycine and 10 g SDS in 1000 ml of deionized water
- pH of solution should be 8.3 and no pH adjustment is required
- Store the buffer at room temperature and dilute to 1x with deionized water before use

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Murine Leukemia Virus (MLV)-based Coronavirus Spike-pseudotyped Particle Production and Infection

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[Abstract] Viral pseudotyped particles (pp) are enveloped virus particles, typically derived from retroviruses or rhabdoviruses, that harbor heterologous envelope glycoproteins on their surface and a genome lacking essential genes. These synthetic viral particles are safer surrogates of native viruses and acquire the tropism and host entry pathway characteristics governed by the heterologous envelope glycoprotein used. They have proven to be very useful tools used in research with many applications, such as enabling the study of entry pathways of enveloped viruses and to generate effective gene-delivery vectors. The basis for their generation lies in the capacity of some viruses, such as murine leukemia virus (MLV), to incorporate envelope glycoproteins of other viruses into a pseudotyped virus particle. These can be engineered to contain reporter genes such as luciferase, enabling quantification of virus entry events upon pseudotyped particle infection with susceptible cells. Here, we detail a protocol enabling generation of MLV-based pseudotyped particles, using the Middle East respiratory syndrome coronavirus (MERS-CoV) spike (S) as an example of a heterologous envelope glycoprotein to be incorporated. We also describe how these particles are used to infect susceptible cells and to perform a quantitative infectivity readout by a luciferase assay.

Keywords: Pseudotyped particle, Murine leukemia virus, Envelope glycoprotein, Coronavirus, Spike

[Background] Viral pseudotyped particles are very useful tools for studying the entry pathways that enveloped viruses use and for generating novel gene-delivery vectors. These synthetic enveloped viruses are derived from a parental virus, usually a rhabdovirus or a retrovirus, which forms the core of the particle that can incorporate in its membrane a wide range of viral envelope glycoproteins from heterologous viruses. Several model viruses such as the retroviral murine leukemia virus (MLV) and human immunodeficiency virus-1 (HIV-1) or the rhabdoviral vesicular stomatitis virus (VSV) have been successfully used to generate viral pseudotyped particles (also named pseudoviruses or pseudovirions). Virus pseudotyping is particularly useful for the following scenarios: (i) to quantify the viral entry process of enveloped viruses using reporter genes like green fluorescent protein (GFP) or luciferase, (ii) to study host cell entry of enveloped viruses that cannot be cultivated in cell culture, (iii) to study entry pathways of risk group (RG) 3 or 4 viral pathogens when biosafety level (BSL) 3 or 4 facilities are not available, (iv) to generate cells stably expressing a specific gene of interest or for specific gene silencing, (v) to produce vectors for gene delivery allowing control over cell tropism. Pseudotyped particles can be used to complement native virus infection assays, especially regarding study of virus entry events.

The protocol described here is highly adaptable both in terms of scale of production and type of envelope glycoprotein that can be incorporated. It has been extensively used in our research on viral entry of various

enveloped viruses, including VSV (Sun *et al.*, 2008), influenza virus (Tse *et al.*, 2014) and coronaviruses (Belouzard *et al.*, 2009; Millet and Whittaker 2014; Millet *et al.*, 2016b). We have successfully used this method to pseudotype viral envelope glycoproteins from all three classes of viral fusion proteins: influenza hemagglutinin (HA, class I), coronavirus spike (S, class I), Ebola glycoprotein (GP, class I), Semliki forest virus (SFV) E1 (class II), and vesicular stomatitis virus (VSV) G glycoprotein (class III). The technique described here is based on work performed by Bartosch and colleagues (Bartosch *et al.*, 2003), and employs the so-called ‘three-plasmid’ co-transfection strategy in which producer HEK-293T/17 cells are co-transfected with the following plasmids: a plasmid allowing expression of MLV retroviral core genes *gag* and *pol* but lacking the MLV envelope glycoprotein-encoding *env* gene, a transfer vector containing a luciferase reporter gene flanked by retroviral regulatory LTR regions and a packaging signal, along with a plasmid allowing expression of the desired envelope glycoprotein. The co-expression of these three plasmids allows synthesis of LTR-flanked reporter gene-containing RNA, MLV-derived proteins and heterologous envelope glycoprotein. During pseudotyped particle formation, which occurs at the plasma membrane, the RNAs containing the LTR-flanked luciferase gene get incorporated into nascent particles formed by assembly and budding of MLV capsid proteins that also recruit heterologous viral envelope glycoproteins. Upon infection in susceptible cells, the pseudotyped virus entry pathway is solely governed by the heterologous virus envelope glycoprotein used. As such, pseudovirions are excellent surrogates to study the entry pathway of enveloped viruses. Once virus entry has occurred, the pseudotyped virus RNAs are released in the cell and the retroviral reverse transcriptase and integrase then reverse transcribe the molecules into double stranded DNA and integrate them into the genome of target cells. Because the sequence that gets integrated only contains the gene encoding the luciferase reporter but none of the MLV genes, the pseudotyped particles are inherently safer as they only allow for one round of infection. After infection, a simple luciferase assay allows quantification of infectivity of the pseudotyped particle studied.

The following protocol can form the basis of useful experiments for the study of the heterologous envelope glycoprotein function during virus entry, for example by performing the infection assay using different infection conditions such as receptor/co-receptor expression in target cells, virus binding time and temperature, pH, endocytosis inhibitors, protease inhibitors, neutralizing antibodies, etc.

Materials and Reagents

1. Cell culture vessels, plates and tubes
 - a. 50 ml Falcon tubes (TrueLine, catalog number: TR2004)
 - b. Cell counting slides with grids (KOVA, catalog number: 87144)
 - c. T75 75 cm² cell culture flasks (TrueLine, catalog number: TR6002)
 - d. 6-well cell culture plates (TrueLine, catalog number: TR5000)
 - e. 24-well cell culture plates (TrueLine, catalog number: TR5002)
 - f. 1.5 ml Eppendorf tubes
 - g. 0.22 µm cell culture medium filtration unit (LPS, catalog number: 1102-RLS)
2. Pseudotyped virus solution filtration
 - a. 0.45 µm filter (Pall, catalog number: 4184)

- b. 10 ml syringes (BD, catalog number: 309604)
- 3. Pipettes and pipettors
 - a. Pipettor set:
 - P1000 (Gilson, catalog numbers: F123602)
 - P200 (Gilson, catalog numbers: F123601)
 - P20 (Gilson, catalog numbers: F123600)
 - b. Stripettor for pipetting with serological pipette (Corning, catalog number: CLS4910-1EA)
Note: This product has been discontinued.
 - c. Sterile serological pipettes:
 - 25 ml (LPS, catalog numbers: TR37129)
 - 10 ml (LPS, catalog numbers: TR37128)
 - 5 ml (LPS, catalog numbers: TR37127)
 - d. Repeater dispenser (Eppendorf, catalog numbers: 4981000.019/022260201)
Note: This product has been discontinued.
 - 10 ml sterile tips (Eppendorf, catalog numbers: 0030089677)
 - 5 ml sterile tips (Eppendorf, catalog numbers: 0030089561)
- 4. Cells
 - a. Human embryonic kidney (HEK) HEK-293T/17 cells (ATCC, catalog number: CRL-11268), maintained in T75 flasks with complete DMEM (DMEM-C)
 - b. Human hepatic Huh-7 cells (National Institutes of Biomedical Innovation, Health and Nutrition, catalog number: JCRB0403), maintained in T75 flasks with DMEM-C
- 5. Transfection plasmids and reagents
 - a. Plasmids pCMV-MLVgag-pol (ampicillin resistance) (Bartosch *et al.*, 2003)
 - b. pTG-Luc (ampicillin resistance) (Bartosch *et al.*, 2003)
 - c. pCAGGS-MERS-S (ampicillin resistance) (Millet and Whittaker, 2014)
 - d. pCAGGS-VSV-G (ampicillin resistance)
 - e. pCAGGS (ampicillin resistance)
 - f. Lipofectamine 2000 (Thermo Fisher Scientific, InvitrogenTM, catalog number: 11668-027)
 - g. Opti-minimal essential medium (Opti-MEM) (Thermo Fisher Scientific, GibcoTM, catalog number: 31985-070)
- 6. Disinfection/decontamination reagents
 - a. Ethanol (95%) (VWR, BDH[®], catalog number: BDH1158-4LP) diluted to 70% with water in spray bottle for surface disinfection
 - b. Paper towels to soak in 70% ethanol and wipe surfaces to disinfect (Georgia-Pacific Consumer Products, catalog number: 23304)
 - c. Bleach solution for decontamination (The Clorox Company, catalog number: Germicidal Bleach)
- 7. Cell culture reagents
 - a. 0.25% trypsin EDTA solution (Mediatech, catalog number: 25-053-CI)
 - b. Dulbecco's phosphate buffered saline (DPBS) with Ca²⁺ and Mg²⁺ (Mediatech, catalog number: 21-030-CV)

- c. Dulbecco's modification of Eagles medium (DMEM) with 4.5 g/L glucose, L-glutamine but without sodium pyruvate (Mediatech, catalog number: 10-017-CV)
- d. Heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, GibcoTM, catalog number: 1614071)
- e. 100× penicillin-streptomycin (PS) solution (Mediatech, catalog number: 30-002-CI)
- f. 1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) solution (Mediatech, catalog number: 25-060-CI)
- 8. Luciferase assay reagents
 - a. Luciferase assay lysis buffer (Promega, catalog number: E1531)
 - b. Luciferin substrate (Promega, catalog number: E1501)
 - c. Sterile water (VWR, catalog number: E476-1L)
- 9. Others
 - a. Cryovials
 - b. Complete DMEM (DMEM-C) (see Recipes)
 - c. Transfection DMEM (DMEM-T) (see Recipes)

Equipment

- 1. Temperature-controlled water bath (Labnet, model: W1106A)
- 2. Biosafety cabinet (Class II-A2) connected to a vacuum aspiration system (Labconco, model: 3440009)
- 3. Inverted light microscope with 10× objective (Nikon Instruments, model: TS100) for checking cell density and health
- 4. Plate rocker in 37°C 5% CO₂ cell culture incubator (Thermo Fisher Scientific, Fisher ScientificTM, model: 13-687-704)
- 5. Plate rocker, room temperature (VWR, model: 40000-300)
- 6. 37°C 5% CO₂ humidified cell culture incubator – Symphony (VWR, model: 98000-368)
- 7. Vortex Genie (Scientific Industries, model: G560)
- 8. Pocket calculator (Sharp Elsimate, Sharp, model: EL-334TB)
- 9. Inverted light microscope with 10x objective (Carl Zeiss, model: Axiovert 200) connected to a CCD camera (PCO, model: Sensicam QE)
- 10. Centrifuge (Eppendorf, model: 5810R)
- 11. GloMax 20/20 luminometer (Promega, model: 2030-100)
- 12. Lab refrigerator set at 4°C (GE Appliances, model: GMR06AAMBRWW)
- 13. Lab freezer set at -20°C (SUMMIT APPLIANCE, model: FS-603)
- 14. Lab freezer set at -80°C (Thermo Fisher Scientific, Thermo ScientificTM, model: UXF30086A)
- 15. Timer (VWR, catalog number: 61161-346)

Software

1. Prism (GraphPad, [version 7](#))

Procedure

Day 1

- A. Cell culture procedures preparations (performed each time cells and viral pseudotyped particles are manipulated) – see Note 1 for basic recommendations regarding cell culture.

1. Pre-warm cell-culture reagents required in 37°C water bath: DMEM-C, DPBS, trypsin.

Note: This is a particularly important step since HEK-293T/17 cells are poorly adherent – see Note 2 for details.

2. Turn on biosafety cabinet for at least 15 min before starting procedures.
3. Disinfect all internal surfaces of biosafety cabinet (except top grills) with 70% ethanol-soaked paper towels.

- B. Cell seeding (performed in biosafety cabinet)

1. Place an 80-90% confluent T75 flask (check by visual inspection using an inverted light microscope) containing HEK-293T/17 cells in sterilized biosafety cabinet.

2. Wash cells twice with 10 ml of pre-warmed DPBS being careful not to dispense liquid directly on cells.

3. Trypsinize cells with 1 ml of pre-warmed trypsin solution.

4. Rock plate gently with hands so the trypsin solution covers the entire surface of cell monolayer.

5. Incubate cells at 37°C 5% CO₂ incubator for 3-5 min avoiding longer incubation times.

Note: It is crucial to avoid long trypsin incubation times because this typically leads to cells clumping – see Note 3.

6. Gently tap sides of flask to detach cells.

7. Place trypsinized flask back into biosafety cabinet.

8. Add 4 ml of warmed DMEM-C to neutralize trypsin activity.

9. Resuspend cells by performing repetitive up-down pipetting using a 5 ml serological pipette.

10. Add 5 ml of DMEM-C to cells and resuspend cells again.

11. Transfer 10 ml of cell suspension to a 50 ml Falcon tube.

12. Vortex cells gently for 30 sec.

13. Transfer 10 µl of cell suspension to a cell counting chamber using a P20 pipettor and appropriate tip.

14. Count cells using the cell counting chamber under an inverted light microscope.

15. Calculate cell density using the calculator.

16. Add DMEM-C to obtain a final cell density of 5×10^5 cells/ml.

17. Resuspend cells by up-down pipetting and gently vortex tube.

18. Seed cells to wells of a 6-well plate by adding 2 ml of cells (1 million cells) per well.

19. Gently rock plate back and forth (avoid swirling movement), sideways and diagonally.
20. Incubate plate at 37°C 5% CO₂ incubator for 16-18 h.

Day 2

C. Transfection (performed in biosafety cabinet)

1. Check for cell confluence using a light microscope – ideally cells should be around 40-60% confluency, see Figure 1.

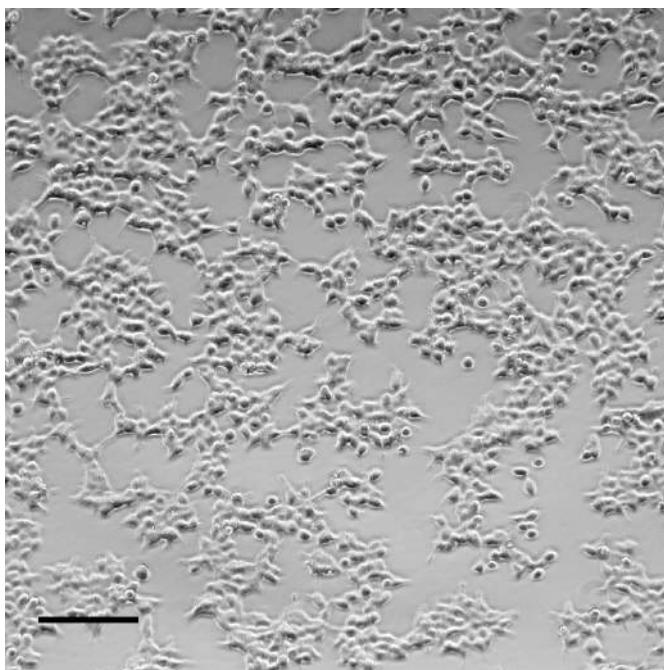


Figure 1. HEK-293T/17 cell confluence before transfection. For efficient transfection and pseudotyped particle production, HEK-293T/17 cell confluence should be around the 40-60% range as shown in the above microscopy picture taken with a Zeiss Axiovert 200 microscope. Scale bar represents 100 µm.

2. Warm Opti-MEM in 37°C incubator.
3. Calculate volumes of plasmid DNA and Opti-MEM required for mix A (the quantities given for 1 well of a 6-well plate can be scaled-up or down depending on needs).

For 1 well of a 6-well plate (1 µg total DNA transfected):

pCMV-MLVgag-pol	300 ng
pTG-Luc	400 ng
Envelope-encoding or empty vector	300 ng
Opti-MEM	to 50 µl

Notes:

- a. *The above DNA ratios can be optimized, if required – see Note 4.*
- b. *Also, this protocol is performed using a 6-well plate format. This can be scaled up or down according to experimental requirements – see Note 5.*

4. Include transfection conditions with co-transfection of pCMV-MLVgag-pol and pTG-Luc with a plasmid for positive control particles (VSV-Gpp, e.g., with pCAGGS-VSV-G) and for negative control particles (Δ envpp, e.g., with pCAGGS empty vector) – see Note 6 for details on pseudotyped particle controls.
5. Calculate volume of Lipofectamine 2000 transfection reagent required for mix B.
For 1 well of a 6-well plate (1:3 ratio used, 1 μ g DNA for 3 μ l Lipofectamine 2000):

Lipofectamine 2000	3 μ l
Opti-MEM	47 μ l

Note: The 1:3 ratio can be optimized, if required – see Note 4.
6. Include 2 ‘safety’ wells in volume calculations for mixes A and B of each transfection condition to avoid running out of solutions when performing the same transfection in multiple wells.
7. Handle Lipofectamine 2000 reagent with care avoiding direct contact with plastic surfaces (tube walls) and always dispense in tubes already containing a liquid solution.
8. Perform mix A and mix B dilutions according to calculations.
9. Incubate at room temperature for 5 min.
10. Use a 1:1 ratio (i.e., 50 μ l each for one well to transfect) of mix A and B and mix solutions by performing multiple up-down pipetting.
11. Incubate at room temperature for 20 min.
12. Place plate of cells to be transfected in incubator and label wells to be transfected with name of viral enveloped used.
13. Aspirate supernatants gently and replace with 1 ml of warmed Opti-MEM.
14. Add 100 μ l of transfection mix to each well in a drop-wise manner.
15. Gently rock plates front and back and side to side.
16. Incubate in 37°C 5% CO₂ incubator for 4-6 h.
17. Pre-warm transfection DMEM (DMEM-T, no antibiotics) in 37°C water bath.
Note: DMEM-T is used here instead of DMEM-C because the transfection reagent increases cell membrane permeability, which leads to enhanced susceptibility to the cytotoxic effects of antibiotics in the cell culture medium – see Note 4.
18. Add gently 1 ml of DMEM-T to each well.
19. Incubate in 37°C 5% CO₂ incubator for 48 h.

Day 4

- D. Harvesting (performed in biosafety cabinet)
 1. Label cryovials with name of pseudotyped virus (envelope glycoprotein used), date of harvest and initials of user.
 2. Collect supernatants in 50 ml Falcon tubes (solutions from multiple wells can be pooled if they were transfected identically).
 3. Spin tubes in centrifuge at 290 \times g for 7 min.
 4. Pass pseudotyped virus solution through a 0.45 μ m filter using appropriate syringe.
 5. Aliquot solution in labeled cryovials (e.g., 1 ml aliquots).

6. Store tubes at -80°C – see Note 7 for more details on storage.
7. Decontaminate with 10% bleach solution all culture vessels, serological pipettes, syringes that have come into contact with pseudotyped virus solutions, with a minimum contact time of 15 min.

Note: This protocol describes how to produce and use pseudotyped particles for infectivity assays.

To estimate quantities of particles produced as well as incorporation of specific viral envelope glycoproteins into particles, additional experiments are required such as Western blot analyses or ELISA assays – see Note 8 for details.

E. Cell seeding for infection (performed in biosafety cabinet)

1. Place an 80-90% confluent T75 flask containing Huh-7 cells in biosafety cabinet.
2. Wash cells twice with 10 ml of warmed DPBS being careful not to dispense liquid directly on cells.
3. Trypsinize cells with 1 ml of warmed trypsin solution.
4. Rock plate gently with hands so the trypsin solution covers the entire surface of cell monolayer.
5. Incubate cells at 37°C 5% CO₂ incubator for 3-5 min avoiding longer incubation times.

Note: It is important to avoid long trypsin incubation times because this typically leads to the clumping of cells as well as cell counting and density problems – see Note 3.

6. Gently tap sides of flask to detach cells.
7. Place trypsinized flask back in biosafety cabinet.
8. Add 4 ml of warmed DMEM-C to neutralize trypsin activity.
9. Resuspend cells by performing repetitive up-down pipetting using a 5 ml serological pipettor.
10. Add 2-3 ml of DMEM-C to cells and resuspend cells again (adjust depending on cell confluence).
11. Transfer cell suspension to a 50 ml Falcon tube.
12. Vortex cells gently for around 30 sec.
13. Transfer 10 µl of cell suspension to a cell counting chamber using a P20 pipettor and appropriate tip.

14. Count cells using the cell counting chamber under a light microscope.
15. Calculate cell density using a pocket calculator.
16. Add DMEM-C to obtain a final cell density of 5×10^5 cells/ml.
17. Resuspend cells by up-down pipetting and gently vortex tube.
18. Seed cells to wells of a 24-well plate by adding 500 µl of cells (2.5×10^5 cells) per well using the repeater dispenser.

Note: The infection is performed in a 24-well plate. This can be scaled up or down depending on experimental requirements – see Note 5 for details.

19. Gently rock back and forth (avoid swirling movement), sideways and diagonally the plate.
20. Incubate plate at 37°C 5% CO₂ incubator for 16-18 h.

Day 5

F. Infection (performed in biosafety cabinet)

1. Check for Huh-7 cell confluence using light microscope (cells should be nearly confluent as shown in Figure 2).

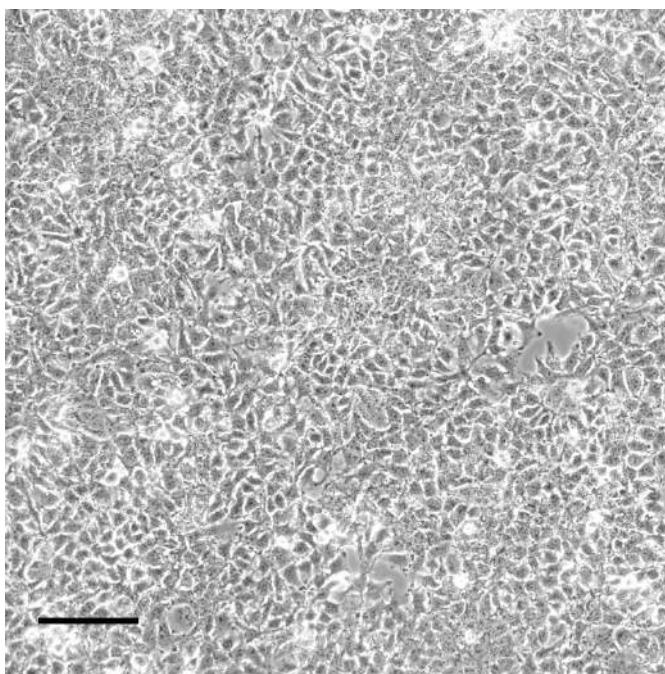


Figure 2. Huh-7 cell confluence before infection with pseudotyped particles. For efficient transduction by pseudotyped particles, Huh-7 cells should be almost completely confluent as shown in the above microscopy picture taken with a Zeiss Axiovert 200 microscope. Scale bar represents 100 µm.

2. Pre-warm DPBS in 37°C water bath.
3. Thaw pseudotyped virus solution vials at room temperature (vials can then be kept on ice).
4. Place the 24-well plate with Huh-7 monolayer in biosafety cabinet.
5. Wash three times with 200 µl of warmed DPBS being careful not to dispense liquid directly on cells.
6. Inoculate wells with 200 µl of pseudotyped virus solution.
7. Include non-infected (n.i.) control conditions by adding 200 µl DMEM-C solution per well.
8. Place the plate on rocker located in 37°C 5% CO₂ cell culture incubator.

Note: Alternatively, cells can be placed in incubator without rocker.

9. Incubate cells for 1-2 h.
10. Pre-warm DMEM-C in 37°C water bath.
11. Add 300 µl DMEM-C using a repeater dispenser and appropriate tip.
12. Incubate at 37°C 5% CO₂ cell culture incubator for 72 h.

Note: This infection time can be optimized if required, see Note 9.

13. Decontaminate with 10% bleach solution all culture vessels and pipette tips, that have come into contact with pseudotyped virus solutions, with a minimum contact time of 15 min.

Day 8

G. Luciferase assay (performed in biosafety cabinet until step G5)

1. Bring luciferase assay lysis buffer (stored at -20°C) and luciferin substrate (stored at -80°C) at room temperature.

2. Place plate containing infected cells in biosafety cabinet and incubate until temperature has equilibrated to room temperature.
3. Dilute to 1× luciferase assay lysis buffer with sterile water.
4. Aspirate gently the supernatants of cells.
5. Add 100 µl of 1× luciferase assay lysis buffer to each well using the repeater dispenser.
6. Incubate on rocker at room temperature for 15 min (after incubation, plate can be opened outside of biosafety cabinet).
7. Turn GloMax 20/20 luminometer on.
8. Prepare clear 1.5 ml Eppendorf tubes for each well to be analyzed by dispensing 20 µl of luciferin substrate solution in each tube.
9. Add 10 µl of lysed cell supernatant to each 20 µl luciferin-containing tube, performing quick up-down pipetting and gentle tube flickering immediately followed by placing the tube in tube holder found in the luminometer.
10. Press 'Measure Luminescence' button on touch screen of luminometer.
11. Record reading of relative luciferase units (RLU).
12. Repeat measurement recording (steps G8-G11) procedure for the other wells.

Data analysis

1. Collect data for each type of pseudovirion (typical values for coronavirus spike-pseudotyped particles (CoVpp) are in the 10⁴-10⁶ RLU range, VSV-Gpp 10⁶-10⁸ RLU range and Δenvpp in the 10² baseline RLU range). If the luminometer is connected to a computer, data can be recorded on an electronic file using supplied software.
2. Include at least duplicate measurements for each infection condition analyzed.
3. Average measurements of independent experiments for statistical significance analysis.
4. Plot data using graph plotting software such as Prism 7 (an example of plotted data is shown in Figure 3).

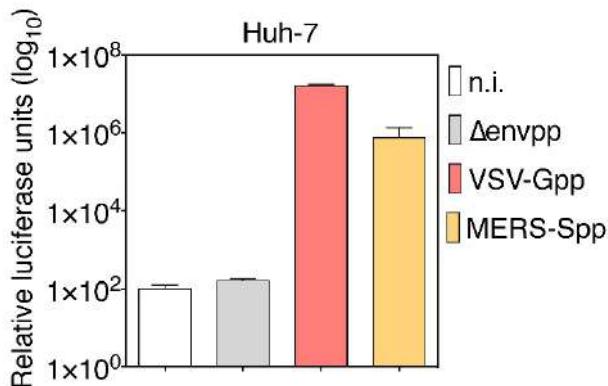


Figure 3. MERS-CoV S-pseudotyped particle infectivity assay in human liver cells (Huh-7).

Displayed are luciferase activity measurements of lysed Huh-7 cells 72 h post-infection for the following conditions: non-infected (n.i.), 'bald' (no envelope) pseudotyped particles (Δenvpp), VSV-

G-pseudotyped particles (VSV-Gpp), and MERS-CoV S-pseudotyped particles (MERS-Spp).

Experiments performed in triplicates and data presented is average relative luciferase units (\log_{10}) of three independent experiments ($n = 3$). Error bars indicate standard deviation (s.d.).

Notes

1. Cell culture and transfection

HEK-293T/17 cells were chosen for transfection and production of pseudotyped particles because they are able to be very efficiently transfected. In this protocol, Huh-7 cells were chosen as target cells for infection by pseudotyped particles because they are very permissive to both native MERS-CoV and MERS-CoV S-pseudotyped particle infection. When producing other types of pseudovirions, we recommend to check what is the most suitable cell line for infectivity assays. As cell lines are the basis for production of pseudotyped particles and for infection assays, it is essential that they are maintained as well as possible by passaging them frequently to avoid having overgrown cell monolayers, using freshly made media as much as possible, and using low passage cells when transfecting or infecting them. Poor cell line health and growth often results in low transfection and infection rates, so it is important to exercise care when handling cells.

2. HEK-293T/17 cell adherence

HEK-293T/17 cells are very useful for this protocol as they typically allow for very high transfection rates. However, these cells tend to adhere poorly to cell-culture treated surfaces, and are easily detached even without trypsin. For this reason, it is important to use pre-warmed Ca^{2+} -containing DPBS when performing washes as these cations help with cell adhesion. Also, use of cell culture surfaces coated with poly-D-lysine (coating solution diluted to 40 $\mu\text{g}/\text{ml}$) are recommended to help with cell adhesion.

3. Trypsin treatment of cells

When trypsinizing cells, particularly Huh-7 cells, we found that incubations of more than 5 min at 37°C 5% CO_2 incubator was deleterious for counting and cell seeding as cells would clump up and would no longer form evenly distributed cell monolayers. We recommend exercising care when trypsinizing cells and avoiding long incubation times with the protease.

4. Transfection recommendations

The ratio of amounts of plasmid DNA used (300 ng pCMV-MLVgag-pol:400 ng pTG-Luc:300 ng envelope-encoding or empty vector) can also be optimized if required (e.g., to increase pseudovirion infectivity), but we recommend to keep the amounts of pCMV-MLVgag-pol and pTG-Luc vectors constant and vary the amount of envelope-encoding vector. Furthermore, in this protocol we have used a 1 μg DNA:3 μl Lipofectamine 2000 ratio for the transfections, which has worked very well for our pseudotyped particle productions. However, depending on the expression plasmids and cell lines used, it may be useful to carry out testing and optimization of this ratio. Also, as the transfection procedure increases cell membrane permeability, it is important to avoid antibiotic treatment of cells during that time. Doing so ensures that the cytotoxicity associated with the transfection remains low.

5. Pseudotyped particle production and infection scaling

The following protocol is performed using 6-well plates for the transfection of HEK-293T/17 producer cells. From one well of a 6-well plate, approximately 2 ml of pseudotyped particle supernatant can be harvested. If more pseudotyped particle solution is required, the same transfection can be performed in multiple wells with the supernatants pooled at the harvesting stage. The protocol can also be scaled up or down depending on needs, by using other culture vessels (such as 10 cm dish or flasks) and by adjusting transfection conditions accordingly. The protocol also details how infection of target cells with pseudotyped particles is performed using 24-well plates. This can also be scaled to the needs of an experiment, for example for high-throughput experiments, the assay can be optimized for infections carried out in 96-well plates by adjusting volumes and using a plate-compatible luminometer.

6. Pseudotyped particle production controls

During production of pseudotyped particles, it is important to include particle production controls, an important step that is detailed in this protocol. Typically, 'bald' or ' Δ env' particles (Δ envpp) are produced by replacing the plasmid encoding the heterologous viral enveloped glycoprotein with an empty vector control. These particles bud out of producer cells, but are devoid of viral envelope glycoproteins. Also, a positive control is also useful to gauge how well the procedure was performed, and typically VSV-G-pseudotyped particles (VSV-Gpp) are used for this. These particles usually reach very high infectivity in many cell types and are thus ideal positive controls.

7. Pseudotyped particle storage

MLV-based pseudotyped particles retain their infectivity well when stored at -80 °C for several months. However, we recommend avoiding multiple freeze-thaw cycles as these can significantly reduce viral infectivity. We recommend aliquoting pseudovirions stocks into small volumes (1 ml) and thawing what is needed for each infection experiment that is being carried out.

8. Quantification of particles produced and envelope glycoprotein incorporation

This protocol details the procedures for pseudotyped particle production and infection. However, it does not describe the methodology for measuring the amount of particles for each batch produced, which is an important step, especially when comparing infectivity of pseudovirions pseudotyped with different types of viral envelope glycoproteins. Such quantifications can be done using several methodologies such as ELISA or Western blot techniques (as done in [Jaume *et al.*, 2011; Millet *et al.*, 2012; Millet and Whittaker, 2014]), allowing estimation of total capsid protein released from producer cells (p30 protein for MLV) or with a direct total particle count and analysis using devices such as Nanosight (Millet *et al.*, 2016a). The Western blot technique also allows to probe for the heterologous envelope protein, which is very useful for assessing efficiency of its incorporation in pseudotyped particles.

9. Incubation times

The incubation times (48 h during production and 72 h for infection assay) have been optimized for MERS-CoV S-pseudotyped particles. These incubation times can be used as guidelines for generating other types of pseudovirions, however, we recommend testing different incubation times to optimize virus production and infectivity.

10. Pseudotyping more than one envelope glycoprotein

This protocol describes the procedures to generate pseudotyped particles that harbor only one kind of envelope glycoprotein, for example the MERS-CoV S protein in the case of MERS-Spp. However, using the co-transfection strategy, it is possible to incorporate more than a single envelope protein. For example, we have successfully generated influenza hemagglutinin (HA) and neuraminidase (NA) pseudotyped viruses (Millet and Whittaker, 2014; Tse *et al.*, 2014).

11. Regulation of enveloped glycoprotein incorporation by C-terminal tail

The C-terminal tail or endodomain of class I viral fusion proteins plays important regulatory roles both for the fusion function but also for the incorporation of the protein into viral particles. It was shown that modifying the C-terminal tail of the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) S protein was associated with enhanced envelope glycoprotein incorporation into retroviral pseudotyped particles (Moore *et al.*, 2004). The modifications included truncations within the C-terminal tail and addition of a portion of the cytoplasmic tail of the HIV-1 envelope glycoprotein. This could be a useful strategy to consider if the heterologous viral envelope glycoprotein fails to efficiently incorporate into pseudotyped particles.

Recipes

1. 500 ml complete DMEM (DMEM-C)
440 ml 1× DMEM
50 ml 10% FBS (heat-Inactivated)
5 ml 1× penicillin-streptomycin (100×)
5 ml 10 mM HEPES (1 M)
Sterile-filter DMEM-C through a 0.22 µm filtration unit
2. 500 ml transfection DMEM (DMEM-T)
445 ml 1× DMEM
50 ml 10% FBS (heat-inactivated)
5 ml 10 mM HEPES (1 M)
Sterile-filter DMEM-T through a 0.22 µm filtration unit

Acknowledgments

We thank Jean Dubuisson for providing the MLV plasmids allowing generation of pseudotyped particles. We also thank all members of the Whittaker lab for helpful discussions. We have successfully used this protocol to generate pseudotyped particles in several publications (Sun, *et al.*, 2008; Belouzard *et al.*, 2009; Millet and Whittaker, 2014; Tse *et al.*, 2014; Millet *et al.*, 2016a and 2016b). Work in the authors' lab is funded by a grant from the National Institutes of Health (Grant R21 AI111085) and is also supported in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under the Centers of Excellence for Influenza Research and Surveillance (CEIRS) Contract No. HHSN272201400005C.

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An Optimized Method for the Production Using PEI, Titration and Neutralization of SARS-CoV Spike Luciferase Pseudotypes

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[Abstract] The protocol outlined represents a cost-effective, rapid and reliable method for the generation of high-titre viral pseudotype particles with the wild-type SARS-CoV spike protein on a lentiviral vector core using the widely available transfection reagent PEI. This protocol is optimized for transfection in 6-well plates; however it can be readily scaled to different production volumes according to application. This protocol has multiple benefits including the use of readily available reagents, consistent, high pseudotype virus production Relative Luminescence Units (RLU) titres and rapid generation of novel coronavirus pseudotypes for research into strain variation, tropism and immunogenicity/sero-prevalence.

Keywords: SARS coronavirus, Lentiviral pseudotype, Virus neutralization

[Background] The production and use of pseudotyped viral particles (PV) is widely established for many viruses, and applications in the fields of serology, surveillance and vaccine development are manifold (Temperton *et al.*, 2015; Carnell *et al.*, 2015). PVs have proven to be powerful tools to study the effects of viral envelope glycoprotein mutations on serological outcomes, viral tropism and immunogenicity studies especially when combined with epitope information. PVs are chimeric viral constructs in which the outer (surface) glycoprotein(s) of one virus is combined with the replication-defective viral 'core' of another virus. PV allow for accurate, sequence-directed, sensitive antibody neutralization assays and antiviral screening to be conducted within a low biosecurity facility and offer a safe and efficient alternative to wildtype virus use, making them exquisitely beneficial for many emerging RNA viruses of pandemic potential. Many of the published protocols require modification of the SARS spike glycoprotein and/or expensive transfection reagents (Temperton, 2009). The protocol presented here utilizes the full-length, non-codon-optimized spike protein in conjunction with the low-cost transfection reagent PEI, making this protocol widely applicable to many stakeholder laboratories. Figure 1 shows a cartoon of the lentiviral SARS-CoV PV production process directed by plasmid co-transfection.

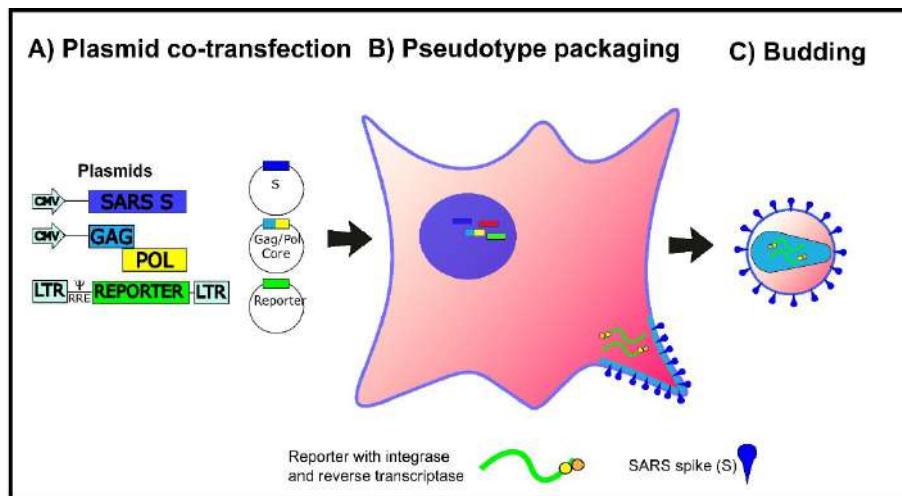


Figure 1. Cartoon representation of the production of SARS pseudotypes. HEK293T/17 cells are transfected with three plasmids, bearing the relevant genes (Lentiviral vector, packaging construct and SARS-CoV spike expression plasmid) for the production of SARS-CoV Spike bearing lentiviral pseudotypes. This figure is modified from Carnell *et al.* (2015).

Materials and Reagents

1. MultiGuard Barrier pipette tips 1-20 and 1-200 µl (Sorenson BioScience, catalog number: 30550T)
2. Nunc™ Cell-Culture Treated Multidishes (6-well) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 140675)
3. Microcentrifuge tube Safe-Lock write-on graduated with lid latch 1.5 ml
4. Sterile syringes (10 ml), Generic
5. Millex-HA 0.45 µm filters (Merck, catalog number: SLHAM33SS)
6. 96-well white plate (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 136101)
7. HEK 293T/17 cells (ATCC, catalog number: CRL-11268)
8. Huh7 cells (Cell Signaling Technology, catalog number: 300156)
9. Plasmids
 - a. Glycoprotein expression plasmid: pCAGGS-SARS-CoV spike
 - b. Lentiviral vector expressing firefly luciferase: pCSFLW
 - c. Second-generation lentiviral packaging construct plasmid: p8.91 (expresses gag, pol and rev)
10. Dulbecco's modified Eagle medium (DMEM) with GlutaMAX (Thermo Fisher Scientific, catalog number: 31966021) supplemented with 10% foetal bovine serum (FBS) (Pan-Biotech, catalog number: P40-37500) and 1% penicillin/streptomycin (P/S) (Pan Biotech, catalog number: P06-07100)

11. Gibco Reduced Serum media Opti-MEM® (Thermo Fisher Scientific, catalog number: 31985047)
12. Branched Polyethyleneimine (PEI) solution at concentration of 1 mg/ml (Sigma-Aldrich, catalog number: 408727).
Note: PEI is dissolved in dH₂O to a concentration of 1 mg/ml and the pH is adjusted to 7 using diluted (1:3) concentrated HCl.
13. Phosphate-buffered saline (PBS)
14. Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific, Gibco™, catalog number: 25300054)
15. Positive control antibody (monoclonal/polyclonal or post-infection serum) that can neutralize the SARS pseudotype
16. Bright Glo™ luciferase assay system (Promega, catalog number: E2650)

Equipment

1. Class II biosafety cabinet (Thermo Fisher Scientific, Thermo Scientific™, model: MSC-Advantage™)
2. Water bath or incubator
3. Pipettes (Gilson, model: PIPETMAN® Classic, P2, P20, P200 and P1000)
4. Optional: BIO-RAD TC20™ Automated Cell Counter (Bio-Rad Laboratories, catalog number: 1450102EDU)
5. Plate centrifuge (ELMI, model: SkyLine CM-6MT)
6. Glomax 96 luminometer (Promega, model: GloMax® 96)
Note: This product has been discontinued.

Procedure

A. Transfection steps

Note: All steps should be carried out in a class II biosafety cabinet to avoid contamination.

Timeline: Transfection–24 h.

1. 293T/17 cells should be subcultured into 6-well plates at a ratio that will deliver 70-90% confluence at the time of transfection. Typically seeding 4×10^5 cells per well will achieve this level of confluency. Example is shown in Figure 2.

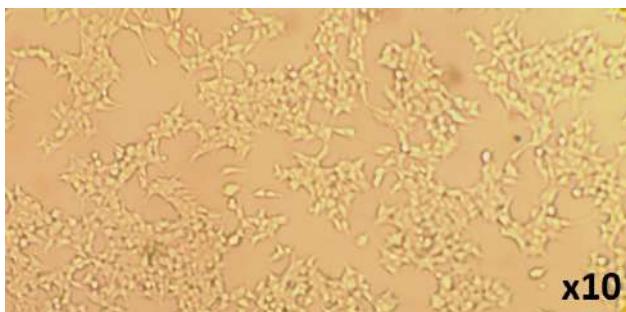


Figure 2. Example of the confluency expected prior to transfection of HEK293T/17 cells

Timeline: Day of transfection.

2. DMEM/10% FBS/1% P/S and Opti-MEM® should be pre-warmed to 37°C using a water bath or an incubator.
3. Prepare two labelled sterile 1.5 ml microcentrifuge tubes (tube 1 and tube 2) for each well of a 6-well plate which will be used for transfections.
4. Add the following plasmid constructs for transfection to tube 1:
pCAGGS-SARS-CoV spike: 450 ng
p8.91-lentiviral vector: 500 ng
pCSFLW: 750 ng
5. Add 100 µl Opti-MEM® to the plasmid DNA mix (tube 1).
6. Add 100 µl Opti-MEM® and 17.5 µl of 1 mg/ml PEI to tube 2.
7. Incubation step: Mix both tubes by gently flicking and incubate for 5 min at room temperature (RT).
8. After incubation, pipette the Opti-MEM®/PEI solution from tube 2 into the Opti-MEM®/DNA solution in tube 1.
9. Incubation step: Incubate the tube at RT for 20 min whilst gently flicking the tube to mix every 3-4 min.
10. Whilst the transfection mix is incubating, the culture media on the 293T/17 cells should be removed and 2 ml of fresh DMEM/10% FBS/1% P/S added per well. It is imperative at this point to add culture media slowly to one side of the well to avoid detaching the adherent cell monolayer.
11. After 20 min incubation, pipette the DNA/Opti-MEM®/PEI solution onto the 293T/17 cells by adding dropwise throughout the total surface area of the well. Swirl the 6-well plate(s) gently to ensure an even dispersal of reagent mix.
12. Incubation step: Incubate the plate at 37°C, 5% CO₂ overnight (o/n). In our hands incubation times of between 12-16 h result in equivalent final PV production RLU titres.

Timeline: 12-16 h post transfection.

13. Post o/n incubation the culture media on the cells should be carefully removed and 2 ml fresh DMEM/10% FBS/1% P/S added. Again, add media slowly to one side of the well to avoid cell detachment.
14. Incubate the 6-well plates at 37°C 5% CO₂ o/n for 32-36 h.

Timeline: 44–52 h post transfection.

15. Supernatant containing the viral pseudotype particles are harvested using a 2.5 ml sterile syringe and subsequently filtered into Falcon or microcentrifuge tubes via a syringe driven Millex HA-0.45 µm filter.
16. Store all filtered supernatants at -80°C until downstream use. It is recommended that supernatant is stored as aliquots to avoid multiple freeze-thaw cycles that may impact viral RLU titres.

Note: Supernatant may be stored at 4°C for up to one week with no detectable loss of RLU titre.

17. Optional step: Additional culture media may be added to cells to allow a second harvest 18-24 h later by adding further DMEM/10% FBS/1% P/S. In this case, extreme care must be taken in initial PV collection (step A15) to avoid damage to the cell monolayer. We have observed that cells in poor health after first harvest result in significantly lower PV production RLU titres upon second harvest.

Note: A control pseudotype virus can be created by following the steps outlined above but omitting the pCAGGS-SARS-CoV spike construct. This produces particles that do not express a viral surface glycoprotein (Delta Env control, Figure 3).

B. Titration steps (Figure 3)

Note: Titration consists of transduction of reporter (in this case firefly luciferase) into target cells mediated by the viral glycoprotein expressed on the viral pseudotype (SARS-CoV spike). Controls for titration are provided via the inclusion of ‘cell only’ and ‘Delta Env’ columns. Control for transduction can be provided via a PV bearing the Vesicular stomatitis virus G protein (VSV-G) which utilizes a ubiquitous receptor which results in high RLU titres in all cell lines tested.

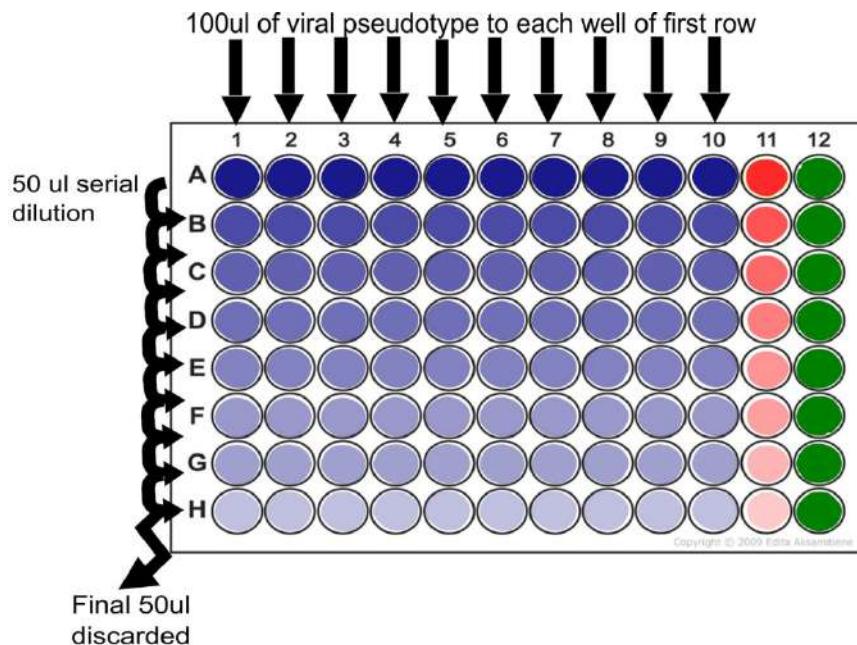


Figure 3. 96-well plate set-up for pseudotype titration. Serial dilution step showing addition of 100 µl of pseudotype virus supernatant to each well of row A and dilution of 50 µl taken from

this well to row B. This process is continued to end of plate (row H) at which point the final 50 μ l is discarded. Delta Env control is indicated in red (column 11) and cell only control is indicated in green (column 12). One set of pipette tips can be used per dilution series (plate).

1. In a 96-well white plate add 50 μ l of DMEM/10% FBS/1% P/S to the entire column of ‘cell only’ control (see Figure 3 column 12).
2. Add 50 μ l of DMEM/10% FBS/1% P/S from row B to H that are to contain PV or Delta-Env control virus.
3. Add 100 μ l of SARS pseudotype virus supernatant to each well of row A (excluding control columns) and add 100 μ l of Delta-Env to column 11 (see Figure 3).
4. Remove 50 μ l from row A virus-containing wells and perform two-fold serial dilutions down all the wells beneath it.
5. With each dilution step use a pipette to mix 8 times by pipetting up and down and taking care not to produce air bubbles.
6. After completing serial dilution the final 50 μ l from the final well of each column should be discarded.

Note: At this point each well should contain 50 μ l of mixed DMEM and PV supernatant.

7. Prepare a plate of susceptible target cells (Huh-7 for SARS PV), preferentially subcultured 1:4 48 h before:
 - a. Remove culture media from plate.
 - b. Wash the plate twice with 2 ml of PBS and discard.
 - c. Add 2 ml of trypsin to the plate to detach cells.
 - d. After cells have detached add 6 ml of DMEM/10% FBS/1% P/S to the plate to quench trypsin activity, and resuspend cells gently.
 - e. Count cells using TC20TM Automated Cell Counter or haemocytometer and add 1×10^4 cells in a total volume of 50 μ l to each well.
8. Centrifuge plate for 1 min at 800 $\times g$ (Rcf) if there are visible droplets on the sides of the wells.
9. Incubate the plate for 48 h at 37°C 5% CO₂.
10. Read plate using Bright GloTM luciferase assay system on a Glomax 96 luminometer (or equivalent).

C. Pseudotype based neutralisation assay (pMN)

Note: pMN is the Inhibition of PV mediated transduction via an antibody (or inhibitor) directed against the SARS glycoprotein.

1. In a 96-well white plate add 50 μ l of DMEM/10% FBS/1% P/S to rows B to H, columns 1-12.
2. Add known amount of antibody (example 5 μ l HN and R sera in Figure 4) into wells of row A, columns 2-10 in a total volume of 100 μ l DMEM/10% FBS/1% P/S. Add known amount (e.g., 5 μ l) of positive and negative antisera into wells A11 and A12 as controls.

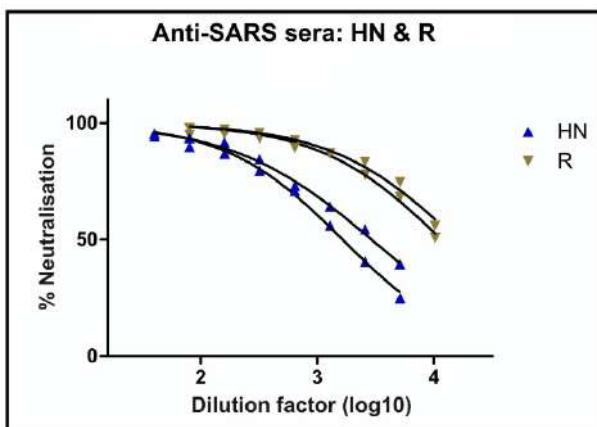


Figure 4. Anti-SARS antibodies (post-infection sera HN and R) neutralization of SARS viral pseudotype entry into Huh7 cells. Two repeats are plotted for each serum sample.

3. Remove 50 μ l from row A wells and perform two-fold serial dilutions down all the wells beneath it.
4. With each dilution step use a pipette to mix 15 times by pipetting up and down and taking care not to produce air bubbles.
5. After completing serial dilution the final 50 μ l from the final well of each column should be discarded.

Note: At this point each well should contain 50 μ l of mixed DMEM and serial dilutions of antibody/inhibitor.

6. Centrifuge plate for 1 min at $800 \times g$ (Rcf) to ensure no inhibitor or liquid is located on the walls of the well.
7. Using data obtained from the titration, calculate the amount of DMEM required to dilute your SARS-PV to obtain 1×10^6 RLU in 50 μ l, with a total volume of 5 ml. For example with an RLU/ml of 1×10^8 , 1 ml of PV should be mixed with 4 ml of DMEM.
8. Mix this diluted PV solution using the multichannel pipette, and aliquot 50 μ l into each well on the plate, with the exception of wells A6-A12 (cell only control). A1-A6 will serve as virus only control.
9. Centrifuge plate for 1 min at $800 \times g$ (Rcf) to ensure no virus is left on the walls of the well.
10. Incubate the plates for 1 h at 37°C 5% CO₂, allowing time for the antibody/inhibitor to bind the SARS glycoprotein.
11. Prepare a plate of susceptible target cells (Huh-7 for SARS PV), preferentially subcultured 1:4 48 h before:
 - a. Remove culture media from plate.
 - b. Wash the plate twice with 2 ml of PBS and discard.
 - c. Add 2 ml of trypsin to the plate to detach cells.
 - d. After cells have detached add 6 ml of DMEM/10% FBS/1% P/S to the plate to quench trypsin activity, and resuspend cells gently.
 - e. Count cells using TC20™ Automated Cell Counter or haemocytometer and add 1×10^4 cells in a total volume of 50 μ l to each well.

12. Incubate the plate for 48 h at 37°C 5% CO₂.
13. Read plate using Bright Glo™ luciferase assay system on a Glomax 96 luminometer (or equivalent).

Data analysis

1. RLU readings are multiplied to RLU/ml by the dilution factor of each well (20×, 40×, 80×, 160×, 320×, 640×, 1,280×, 2,560×). The mean of all 8 RLU/ml values is used as the final value reported for that column in the titration step. A linear relationship should be observed between RLU values and PV dilution, with values decreasing by 50% after each 1:2 dilution. Care should be taken to check this linear relationship before multiplication, as this inherently can lead to false positives despite lack of PV or working PV (Table 1).

Table 1. Analysis of PV titration data. RLU values are multiplied to give an RLU/ml value (example highlighted in green) giving RLU/ml values for each of the dilution points. The mean/average is then calculated from all 8 dilution points. Care must be taken to observe a linear relationship between dilution factor (X factor) and RLU, or multiplication can lead to inflated RLU/ml values (example highlighted in yellow).

X factor	SARS-S	VSV-G	Virus X
20	5.58E+05	7.17E+08	3.05E+03
40	2.90E+05	5.63E+08	1.10E+03
80	1.43E+05	3.79E+08	6.29E+03
160	6.17E+04	2.16E+08	8.80E+02
320	3.10E+04	1.36E+08	5.43E+03
640	2.41E+04	6.18E+07	3.39E+03
1280	7.15E+03	3.06E+07	1.96E+03
2560	3.62E+03	1.44E+07	3.33E+03
RLU/ml	1.12E+07	1.43E+10	6.11E+04
	1.16E+07	2.25E+10	4.40E+04
	1.14E+07	3.03E+10	5.03E+05
	9.87E+06	3.46E+10	1.41E+05
	9.91E+06	4.35E+10	1.74E+06
	1.54E+07	3.96E+10	2.17E+06
	9.16E+06	3.92E+10	2.51E+06
	9.26E+06	3.69E+10	8.54E+06
Average RLU/ml	1.10E+07	3.26E+10	1.96E+06

2. Protocol read-out and titration results (Figure 5).

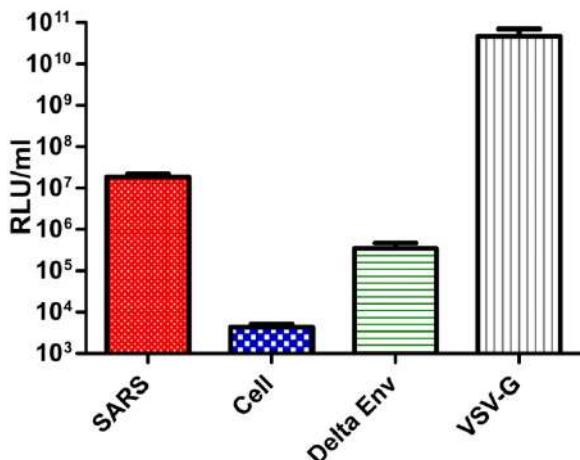


Figure 5. Results of pseudotype production RLU titres using optimized transfection protocol. SARS; PV with SARS-CoV Spike on surface, Cell; cell only control, Delta Env; PV with no surface glycoprotein, VSV-G; PV with VSV-G on surface.

3. The protocol outlined provides a rapid and consistent method for the generation of high-titre viral pseudotype particles expressing the SARS-CoV spike protein suitable for further downstream R&D applications. Efficient knock-down (neutralization) of SARS-CoV pseudotype virus entry using two post-infection sera (HN and R) demonstrates potential utility for vaccine immunogenicity and mAb/antiviral screening. The use of readily available reagents should facilitate increased reproducibility.

Acknowledgments

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Protein Structure Analysis Method of SARS-COV-2 M Protein for Possible Clues Regarding Virion Stability, Longevity and Transmission

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[Abstract] The Severe Acute Respiratory Syndrome Coronavirus 2 or SARS-COV-2 has been the cause of a global pandemic in 2020. With the numbers infected rising well above a 1.9 million and confirmed deaths above 122,000 as of 15th April 2020, it has become the paramount health concern for the global community at present. The SARS-COV-2 genome has since been sequenced and its predicted proteome identified. In this study, we looked at the expected SARS-COV-2 proteins and compare them to its close relative, the Severe Acute Respiratory Syndrome-Related Coronavirus. In particular we focussed on the M protein which is known to play a significant role in the virion structure of Coronaviruses. The rationale here was that since the major risk factor associated with SARS-COV-2 was its ease of spread, we wished to focus on the viral structure and architecture to look for clues that may indicate structural stability, thus prolonging the time span for which it can survive free of a host. As a result of the study, we found some rather interesting differences between the M protein for SARS-COV-2 and the SARS-CoV virus M protein. This included amino acid changes from non-polar to polar residues in regions important for anchoring the protein in the envelope membrane.

Keywords: Proteomics, Systems Biology, M protein, SARS-CoV-2, SARS-CoV

[Background] The SARS-COV-2 Novel Corona virus or Severe Acute Respiratory Syndrome Coronavirus 2 has been the cause of a worldwide pandemic in 2020. The novel strain, originating from Wuhan, China has as of 25th April World Health Organization report, infected over 2.7 million people and led to the deaths of over 187,000 individuals worldwide (Coronavirus disease (COVID-2019) situation report-96, 2020). It has led to considerable disruptions in the economic and psychological welfare of populations, on top of the continuously increasing loss of life (Chen *et al.*, 2020). Efforts at identifying possible solutions to combating the virus have thus far ranged from basic precautionary awareness campaigns to the suggestions of nucleotide based drugs currently being tested for other diseases as possible therapeutic agents (Martinez, 2020). The genome of the SARS-COV-2 novel coronavirus has been decoded and its expected proteome already established (RefSeq ID: [NC_045512.2](#)). However up until this point, to the best of our knowledge not much progress has been made in terms of identifying unique molecular features that are exclusive to this strain of the Coronavirus. Hence, in this study we made an attempt at studying the protein sequences of a relevant SARS-COV-2 architectural protein in order to identify unique advantageous features. We chose to focus our efforts on the M Protein as it is

the most abundant protein in the viral nucleocapsid and is believed to be responsible for maintaining the virion in its characteristic shape (Nal, 2005). This coincides with our investigative rationale; is there any particular feature that aids the spread of this strain. The percentage of infected patients who have died from this virus has been estimated to be approximately 6.9% percent as of this writing (WHO, 2020b). Furthermore the primary susceptible groups are the immunocompromised. This leads us to believe that the primary focus needs to be on the mechanism of spreading, as the rapid rates at which this strain has infected communities across the world has arguably been its most dangerous aspect. The coronaviruses are known to contain four main structural proteins, the S, M, E, and N proteins (Fehr and Perlman, 2015). All of whom have been identified in the predicted proteome of SARS-CoV-2. The S protein is the spike protein, responsible for gaining entry into the ER following infection (Delmas and Hurst, 2009; Beniac *et al.*, 2006). The N protein resides inside the nucleocapsid and is an RNA binding protein (Chang *et al.*, 2005; Hurst *et al.*, 2009). The M and E proteins are both transmembrane proteins. The M protein, a 3 pass transmembrane protein is particularly significant as it is believed to be responsible for maintaining and giving the virion its shape (Armstrong *et al.*, 1984).

Software

1. NCBI Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)
2. SWISS-MODEL (<https://swissmodel.expasy.org/interactive>)

Procedure

A. Selecting Target Protein

Target protein was selected based on its importance to virion structure. M Protein is the most abundant protein in viral nucleocapsid. It plays a key role in maintenance of virion shape and architecture. Hence it was chosen as the target of analysis based on its unique features may play a crucial role in facilitating the survival and transmission of the virion.

B. Sequence Alignment

1. Refseq M protein amino acid sequences were obtained from NCBI Protein.
2. SARS-CoV-2 and SARS-CoV M protein sequences were aligned using Blastp. All parameters were kept as default.
3. Amino acid substitutions between the two proteins were checked and correlated with their functional significance to the protein.

C. Protein Modelling

3D structures for both proteins were predicted using SWISS-MODEL. It should be noted that reference structures exist for the SARS-CoV M protein but we chose to put it through SWISS-MODEL so we could compare the two proteins after they had been modelled by the same tool, so as to eliminate any discrepancy resulting from use of different software.

Data analysis

1. We obtained the protein sequences for the SARS Related Coronavirus as well as SARS-COV-2 and did a multiple sequence alignment using NCBI Blast (Altschul *et al.*, 1990). Figure 1 shows the results of the alignment. Afterwards we took the sequences from the both proteins and attempted to predict their structures using SWISS-MODEL (Biasini *et al.*, 2014).

As Figure 1 shows, there were a total of 20 mismatches and 1 gap between the two sequences. What caught our eye most was the fact that there were multiple amino acid substitutions in the transmembrane domains where bulkier non-polar amino acids in the SARS-CoV gene were replaced by more polar or less bulky ones in the SARS-COV-2 protein. In addition, we found a serine inserted into position 4 of the SARS-COV-2 M protein which its SARS Related Coronavirus counterpart did not have.

Query: YP_009724393.1 membrane glycoprotein [Severe acute respiratory syndrome coronavirus 2] Query ID: lcl|Query_21953 Length: 222

>NP_828855.1 matrix protein [Severe acute respiratory syndrome-related coronavirus]
Sequence ID: Query_21955 Length: 221
Range 1: 1 to 221

Score:416 bits(1070), Expect:3e-155,
Method:Compositional matrix adjust.,
Identities:201/222(91%), Positives:214/222(96%), Gaps:1/222(0%)

Query 1	MADSNGTITVEELKKLLEQWNLVIGFLFLTWICLLQFAYANRNRFYIIKLIKFLWLLWPV	60
Sbjct 1	MAD NGTTITVEELK+LLEQWNLVIGFLFL WI LLQFAY+NRNRFLYIIKLIK+FLWLLWPV	59
Query 61	TLACFVLAAVYRINWITGGIAIAMACLVGLMWLSYFIASFRLFARTRSMSFNPETNILL	120
Sbjct 60	TLACFVLAAVYRINW+TGGIAIAMAC+VGLMWLSYF+ASFLFARTRSMSFNPETNILL	119
Query 121	NVPLHGTILTRPLLESELVIGAVILRGHLRIAGHHLGRCDIKDLPKEITVATSRTLSYYK	180
Sbjct 120	NVPL GTI+TRPL+ESELVIGAVI+RGHLR+AGH LGRCDIKDLPEITVATSRTLSYYK	179
Query 181	LGASQRVAGDSGFAAYSRYRIGNYKLNTDHSSSSDNIALLVQ	222
Sbjct 180	LGASQRVGTDSGFAAYNRYRIGNYKLNTDHAGSNDNIALLVQ	221

Figure 1. BLAST Multiple Sequence Protein alignment of SARS-COV-2 M Protein with SARS-CoV M Protein

Serine provides an extra OH group near the N terminal end of the protein which we know makes up its ectodomain that is glycosylated (Nal *et al.*, 2005). Coming over to the transmembrane regions, at position 33 the SARS-COV-2 has a Cysteine, the equivalent position on SARS-CoV is occupied by a Methionine. At position 30, SARS-COV-2 contains a Threonine, the corresponding position on SARS-CoV has an Alanine. Then at position 76 on the second

transmembrane domain, the SARS-CoV protein contained a valine, compared to an Isoleucine on the SARS-CoV-2 protein. Starting from the latter, Isoleucine has a bulkier sidechain compared to valine, which in the hydrophobic interior of an envelope membrane could produce more hydrophobic interactions with the fatty acid chains of the lipid bilayer, possibly stabilizing the membrane to a greater degree. Furthermore, this substitution occurs within the Mn2 epitope that binds to cytotoxic T lymphocytes (Liu *et al.*, 2010). The previous couple of changes, the Metto Cys and Ala to The substitutions would seem more curious. Both would replace non-polar sidechains with polar groups. This may seem counterintuitive for stabilizing a membrane structure, however crucially perhaps, this could theoretically allow for inter-chain bonds between protein molecules.

The OH group introduced by Threonine could contribute to hydrogen bonding, while the thiol group introduced by Cysteine could give rise to a disulphide bond. We believe this may hold special importance, especially since our modelling run predicted the SARS-CoV-2 M Protein to form homo-trimers, whereas the SARS-CoV M protein is known to form homo-dimers (Godet *et al.*, 1992). The latter was backed up when ran the SWISS-MODEL tool on the amino acid sequence from the SARS-CoV protein for which the first predicted structure was a homo-dimer. Figures 2A and 2B show the predicted structures for SARS-CoV-2 and SARS-CoV M Protein respectively.

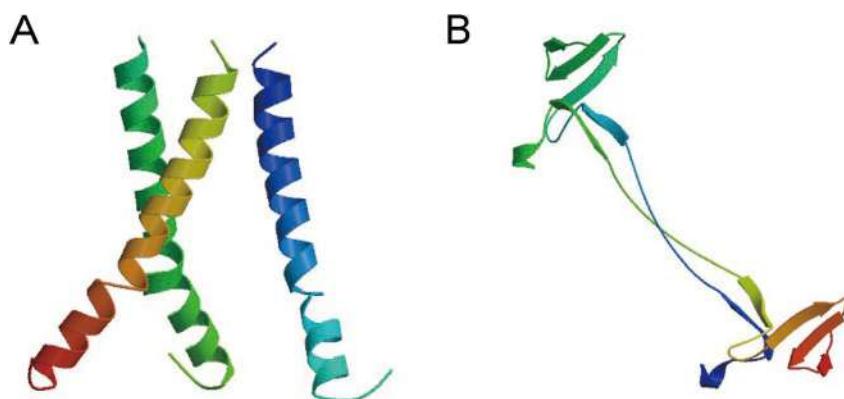


Figure 2. A: SARS-CoV-2 M Protein predicted 3D structure. B: SARS-CoV M Protein 3D structure.

2. Lastly we looked up known protein domains and epitopes within the SARS Related Coronavirus M Protein and scrutinized for any changes in those regions for the SARS-CoV-2 M Protein. The most significant epitope we found and considered was the Mn2 epitope that binds to human Cytotoxic T Lymphocytes (Liu *et al.*, 2010).

Discussion

The goal of this study was to design a simple method for analysing a viral structural protein and understanding the role it may play in the survival and stability of the virion. The two main requirements for using this protocol is A) a structural protein that is known to play a significant

role in viral architecture and B) a close relative of the virus that expresses the same protein but is known to be less easily transmittable. If both of these are available, using this method can provide a simple framework for making an initial deduction on the importance of said structural protein. As for the results of this particular study, they lead us to a possible answer to the question we posed at the very start. We believe there might be a possibility of additional bonding interactions in the SARS- COV-2 M protein that allows its structure to remain more stable and survive for longer. In general, it is what one would expect to happen with the introduction of additional protein-protein bonding, as per the usual biochemical make-up of lipid bilayer membranes and transmembrane proteins that pass through them (Alberts, 2008). The Serine at the N terminus is another interesting observation. The N terminal of this protein is expected to be on the outside of the envelope (Nal *et al.*, 2005) and possibly be exposed to the air and outside interactions.

Something that has come up in recent news reports is the ability of the SARS-COV-2 viral particles to survive for longer on metal surfaces, although the actual study has not yet been published (Kumar and Salzman, 2020). Metals have lattice structures where the positive ions are in the middle and the electrons delocalised and free to move about (Atkins and Shriver, n.d.). A polar group such as an –OH where the hydrogen atom would likely have a partial positive charge, may open the possibility for some kind of bio-electrostatic association that could help the virion to adhere to these surfaces. Although we are not sure as to how or if it could preserve the architectural integrity of the virion.

The major difference between the SARS-CoV and the SARS-COV-2 outbreaks is the spread of the latter. SARS-CoV only effected 26 counties, with around 8000 confirmed cases (WHO | SARS (Severe Acute Respiratory Syndrome), 2020). Granted, this needs to factor in the increased air travel in recent times, which, as per Statista is almost double it was in 2003 when the SARS epidemic happened. The information for that can be found here; <https://www.statista.com/statistics/564769/airline-industry-number-of-flights/>. Nonetheless, based on evidence and our molecular level analysis, we believe the M protein should be a candidate into future investigations that could shed more light on how the SARS-COV-2 virus survives and functions.

Competing interests

The authors declare no competing interests.

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Optimised Method for the Production and Titration of Lentiviral Vectors Pseudotyped with the SARS-CoV-2 Spike

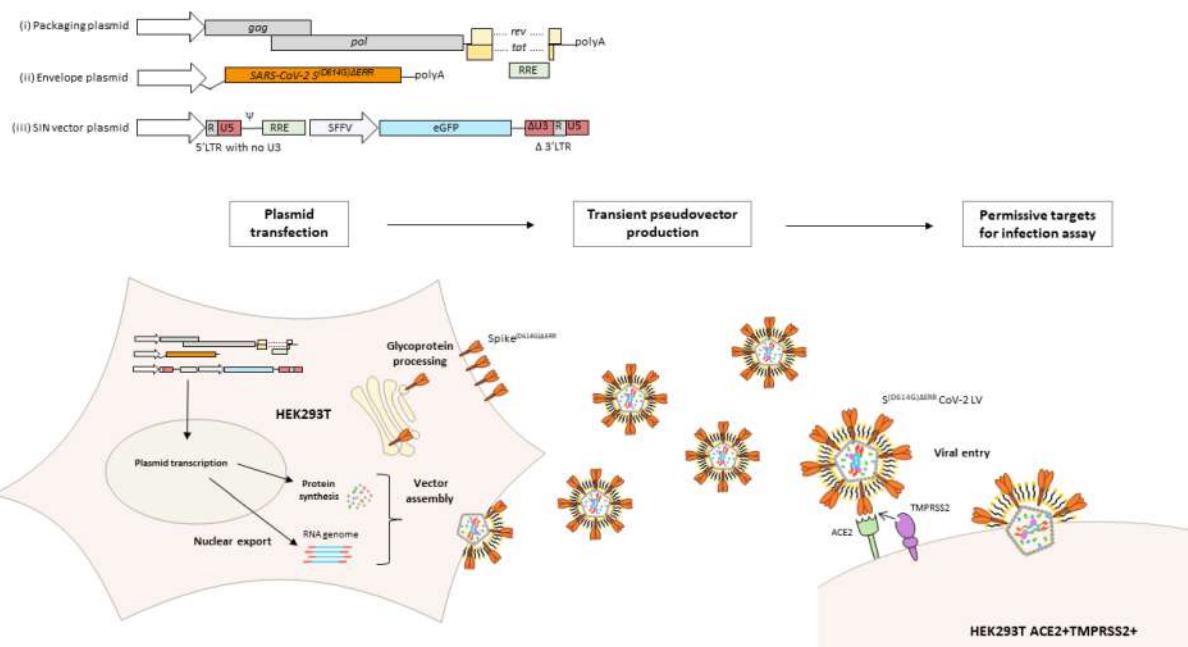
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[Abstract] The use of recombinant lentivirus pseudotyped with the coronavirus Spike protein of SARS-CoV-2 would circumvent the requirement of biosafety-level 3 (BSL-3) containment facilities for the handling of SARS-CoV-2 viruses. Herein, we describe 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.

Graphic abstract:



Production and quantification of lentiviral vectors pseudotyped with the SARS-CoV-2 Spike glycoprotein.

Keywords: SARS-CoV-2 glycoprotein, Lentiviral vector pseudotyping, Spike ER retention signal, D614G mutation, Stable ACE2/TMPRSS2 HEK293T/17 cells

[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 cm² 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 ≥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
Lentiviral transfer vector encoding eGFP downstream of an SFFV promoter in a pCCL backbone.
 - b. Second-generation lentiviral packaging plasmid: pCMVR8.74 (Addgene, catalog number: 22036)
 - c. Glycoprotein expression plasmid: pCDNA-SARS-CoV-2-S^{(D614G)ΔERR}
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 (ΔERR).
15. Iscove's Modified Dulbecco's Medium (Merck, Sigma-Aldrich, catalog number: I3390) supplemented with 2mM 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 LSRIFortessa™ 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

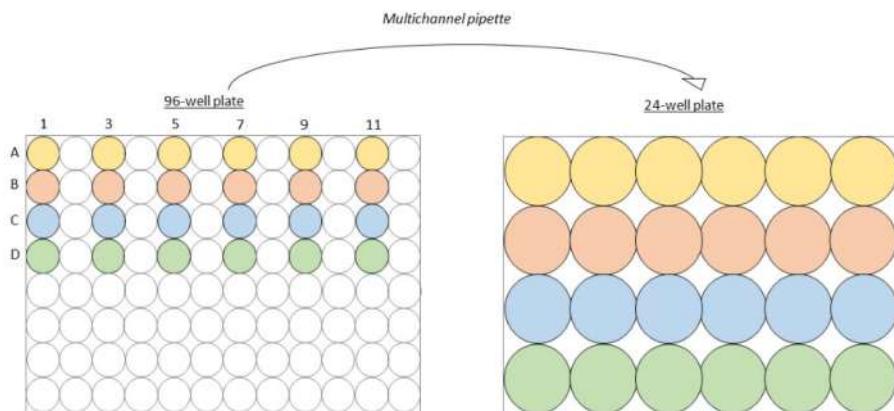


Figure 1).

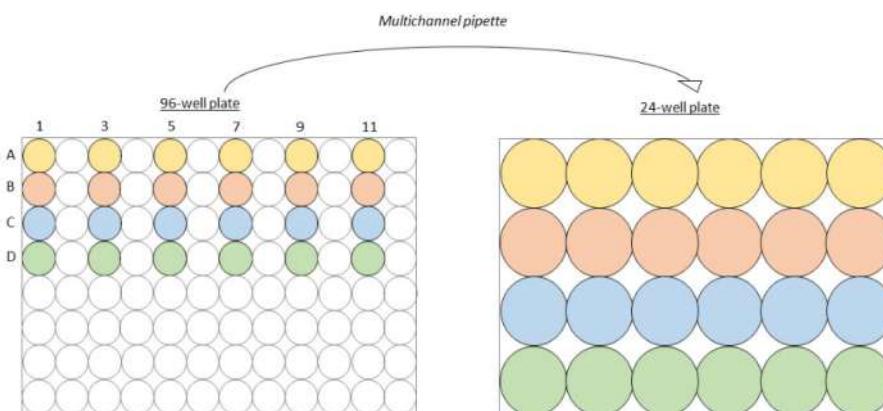
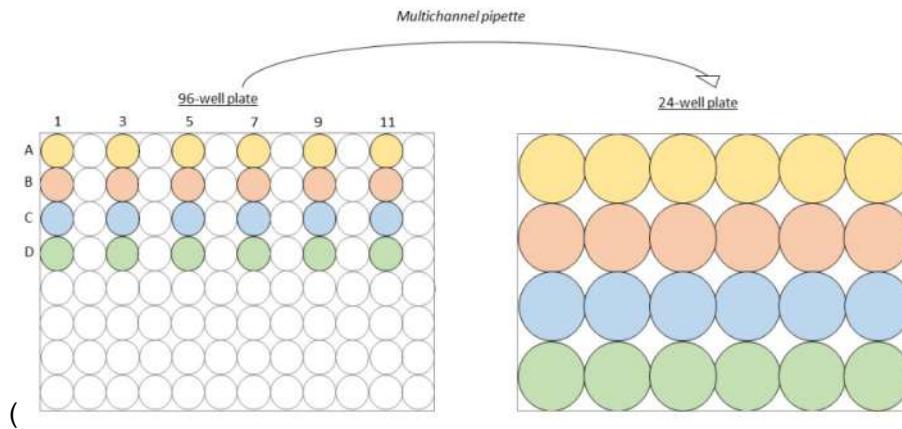


Figure 1. Transfer between 96-well and 24-well plates

- 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



- g. Figure 1).
- h. Centrifuge the plate at 1,000 × g for 20 min to enhance transduction by spinoculation.
- i. 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

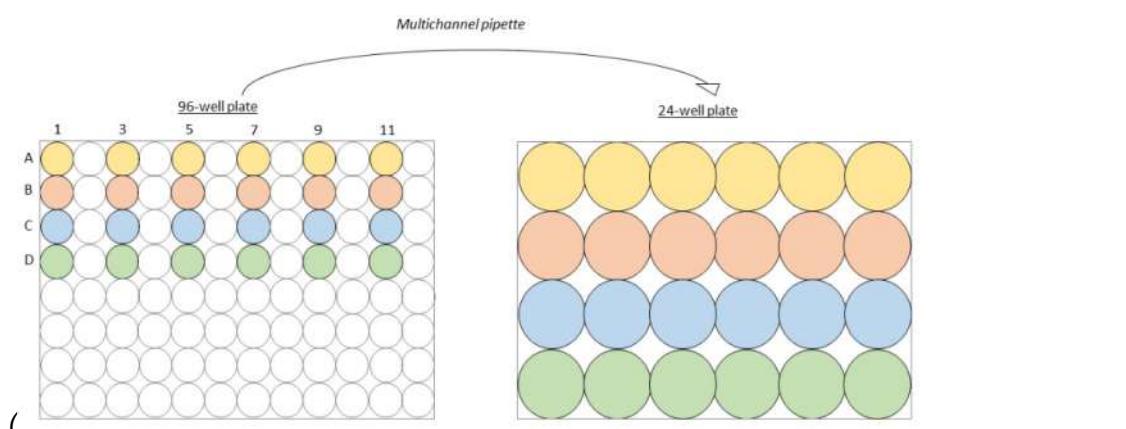


Figure 1).

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 μ L PBS/well and centrifuge plate at $400 \times g$ for 5 min.
8. In the meantime, prepare a master mix for Fixable Viability Dye eFluorTM 780 (0.1 μ L + 99.9 μ L 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 μ L /well staining master mix.
11. Incubate at room temperature for 30 min in the dark.
12. Add 150 μ L PBS/well and centrifuge plate at $400 \times g$ for 5 min.
13. Discard PBS, blot plate, and resuspend cells in 50 μ L of PBS using P200 tips.
14. Acquire plate on flow cytometer with the following bandpass filters:
 - i. RL2 780/60 for Fixable Viability Dye eFluorTM 780.
 - ii. 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

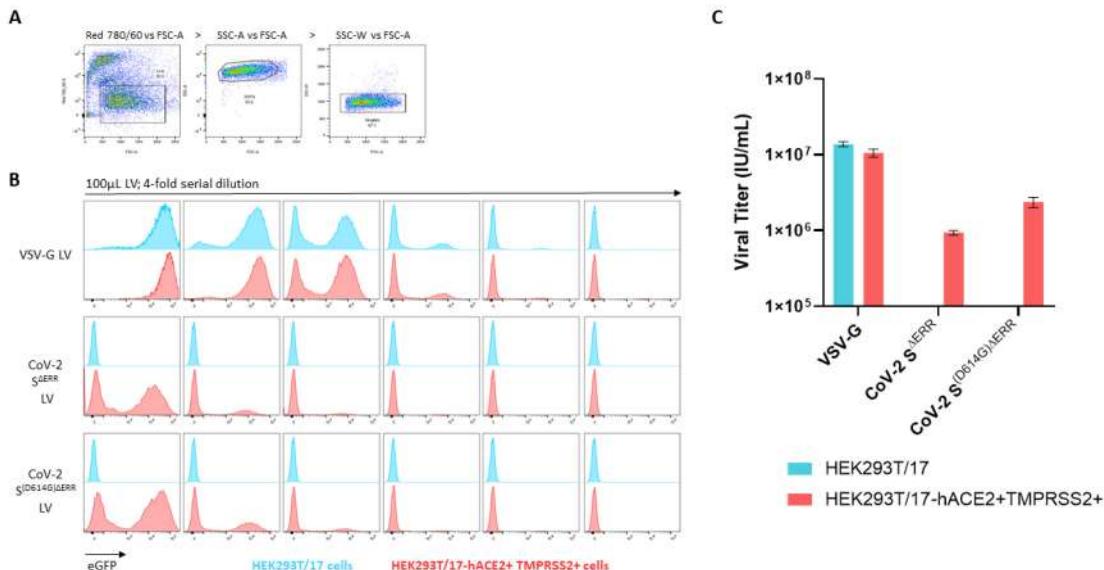
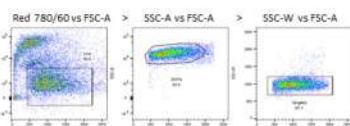


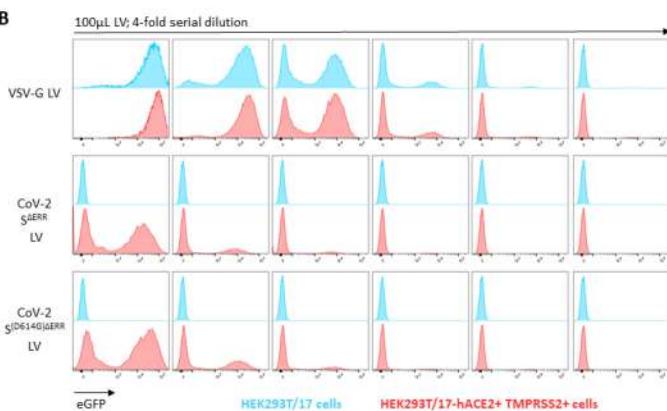
Figure 2A, starting with the live cell gate, followed by the forward versus side scatter gate, and the singlets identification. Singlets are then plotted as histograms to identify the percentage of eGFP-positive cells

(

A



B



C

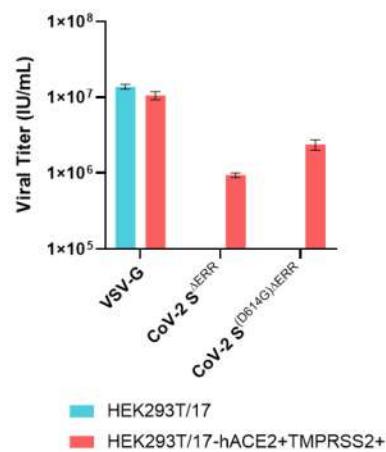


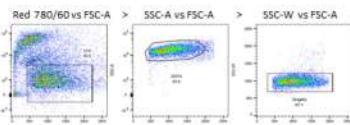
Figure 2B).

2. Determine the percent of transduced cells in each well

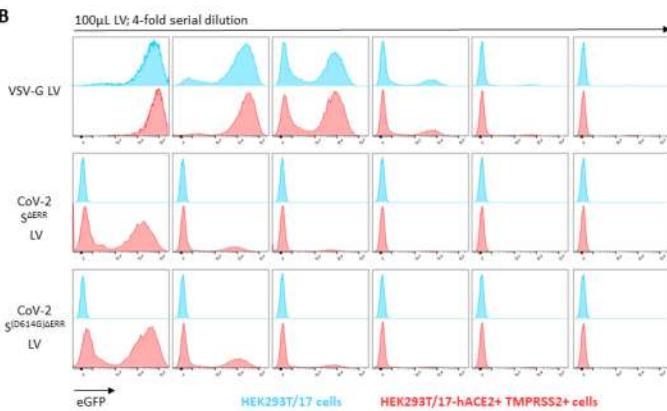
When calculating titers, only transduction efficiencies of less than 20% of transduced cells should be considered. This method assumes one integration per cell. Using >20% risks counting cells with multiple integration sites which leads to an underestimation of the titer. Transduction efficiencies between 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%

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A



B



C

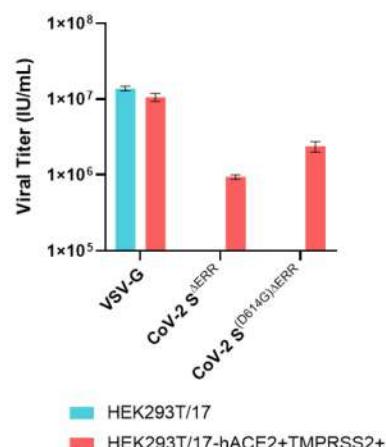


Figure 2C). Titration calculations are presented in Table 1 as an example.

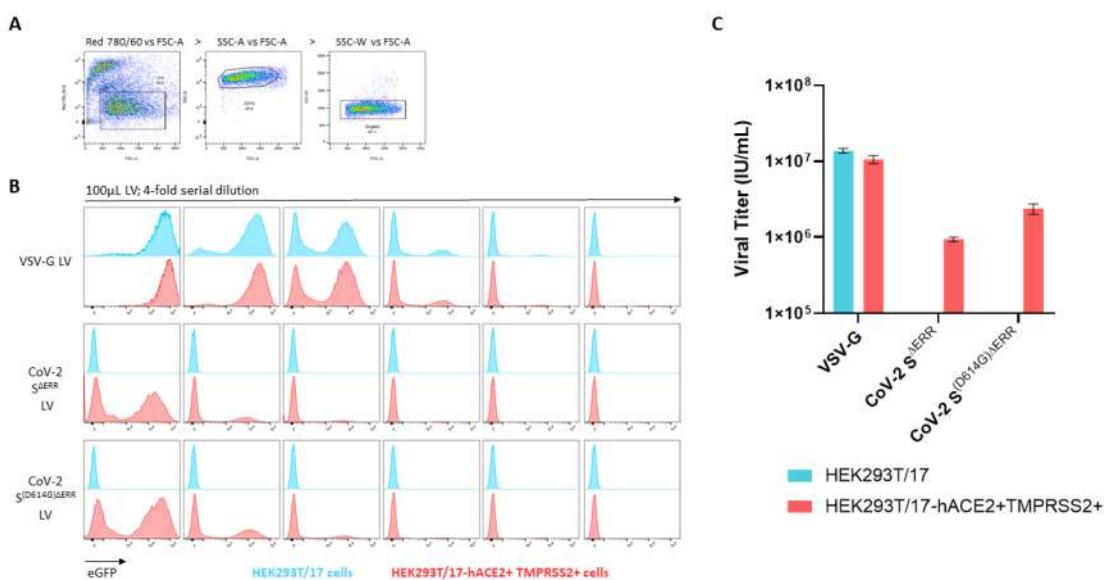


Figure 2. Flow cytometric analysis of pseudotyped lentiviral vectors on stable HEK293T/17-hACE2+TMPRSS2+. (A) Gating strategy starting with live (eF780) vs. FSC-A to identify live cells, followed by SSC-A vs. FSC-A to identify HEK293T/17 cells, and by SSC-W vs. FSC-A for singlets identification. (B) Transiently produced VSV-G LV, S^{ΔERR} CoV-2 LV, and S^{(D614G)ΔERR} CoV-2 LV were serially diluted from 100 μl 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 μg/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 ±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^{ΔERR} LV, and CoV-2 S^{(D614G)ΔERR} 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 (μ l)	HEK293T/17-hACE2+ TMPRSS2+						HEK293T/17					
	VSV-G		CoV-2 S $^{\Delta\text{ERR}}$		CoV-2 S $^{(\text{D}614\text{G})\Delta\text{ERR}}$		VSV-G		CoV-2 S $^{\Delta\text{ERR}}$		CoV-2 S $^{(\text{D}614\text{G})\Delta\text{ERR}}$	
	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+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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Production of Recombinant Replication-defective Lentiviruses Bearing the SARS-CoV or SARS-CoV-2 Attachment Spike Glycoprotein and Their Application in Receptor Tropism and Neutralisation Assays

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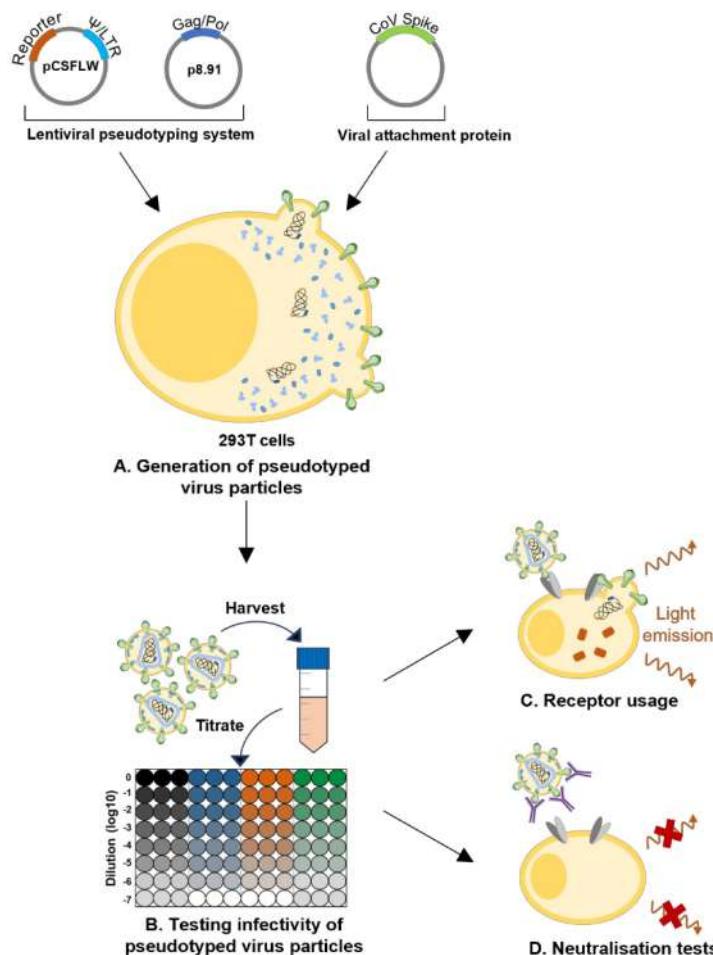
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[Abstract] For enveloped viruses, such as SARS-CoV-2, transmission relies on the binding of viral glycoproteins to cellular receptors. Conventionally, this process is recapitulated in the lab by infection of cells with isolated live virus. However, such studies can be restricted due to the availability of high quantities of replication-competent virus, biosafety precautions and associated trained staff. Here, we present a protocol based on pseudotyping to produce recombinant replication-defective lentiviruses bearing the SARS-CoV or SARS-CoV-2 attachment Spike glycoprotein, allowing the investigation of viral entry in a lower-containment facility. Pseudoparticles are produced by cells transiently transfected with plasmids encoding retroviral RNA packaging signals and *Gag-Pol* proteins, for the reconstitution of lentiviral particles, and a plasmid coding for the viral attachment protein of interest. This approach allows the investigation of different aspects of viral entry, such as the identification of receptor tropism, the prediction of virus host range, and zoonotic transmission potential, as well as the characterisation of antibodies (sera or monoclonal antibodies) and pharmacological inhibitors that can block entry.

Graphic abstract:

SARS-CoV and SARS-CoV-2 pseudoparticle generation and applications



SARS-CoV and SARS-CoV-2 pseudoparticle generation and applications.

Keywords: SARS-CoV-2, SARS-CoV, Pseudotyped virus, Tropism, Neutralisation

[Background] Pseudoparticles are replication-defective viral particles obtained through expression of viral envelope glycoproteins on the surface of a recombinant virus, which provides the core components of the particle. Vesicular stomatitis virus (VSV), a rhabdovirus, and two lentiviruses – human immunodeficiency virus-1 (HIV-1) and murine leukaemia virus (MuLV) – are commonly used as viral vectors for pseudotyping (Takada *et al.*, 1997; Wool-Lewis and Bates, 1998; Sharkey *et al.*, 2001; Negrete *et al.*, 2005; Grehan *et al.*, 2015; Thakur *et al.*, 2021). In our recent study, we successfully used a lentiviral-based system to study the interaction of severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 Spike (S) protein with its cellular receptor, angiotensin converting enzyme 2 (ACE2) (Conceicao *et al.*, 2020).

SARS-CoV-2, the etiological agent of the ongoing COVID-19 pandemic, is a highly pathogenic betacoronavirus that requires handling at BSL-3 facilities, which are not always available in research laboratories. To allow work with SARS-CoV and SARS-CoV-2 at lower containment, the generation of

viral particles pseudotyped with the Spike protein represents a safe and appealing surrogate. This technique allows (i) dissection of viral entry pathways, (ii) investigation of host cell susceptibility and tropism of the angiotensin converting enzyme 2 (ACE2) receptor, (iii) examination of inter-species transmission, (iv) assessment of the neutralising antibody responses in immunogenicity and sero-epidemiological studies, and (v) efficacy assessment of small-molecule inhibitors that block viral entry. Notably, this technique has been applied to viral glycoproteins from a wide variety of viruses such as influenza hemagglutinin (Bertram et al., 2010), Nipah virus fusion and attachment proteins (Thakur et al., 2021), Ebola virus glycoprotein (Simmons et al., 2003), Chikungunya virus E1 (Salvador et al., 2009), hepatitis C virus E2 proteins (Hsu et al., 2003), and VSV glycoprotein (DePolo et al., 2000).

To generate lentiviral-based pseudoparticles of HIV-1, cells are co-transfected with the following plasmids: (i) HIV-1 packaging plasmid encoding for the core genes *Gag* and *Pol*, (ii) the transfer plasmid that encodes a firefly luciferase reporter gene flanked by HIV-1 regulatory LTR regions and the packaging signal, and (iii) a third plasmid encoding for the heterologous viral glycoprotein. Pseudoparticles possessing the viral glycoprotein of interest on their surface are assembled at the cellular membrane, from which they bud (Zufferey et al., 1997). Upon infection, the luciferase gene encoded by the lentivirus genome is expressed, allowing accurate quantification of viral entry.

Materials and Reagents

1. 50 ml Falcon tubes (VWR International, catalog number: 734-0448)
2. Clear bottom 6-well tissue-culture treated plate (Scientific Laboratory Supplies, FalconTM, catalog number: 353046)
3. Pipette tips (STARLAB, catalog numbers: S1110-3700 [10/20 µl XL Graduated TipOne[®]]; S1111-1206-C [200 µl Yellow Bevelled TipOne[®] Tip]; S1112-17200 [1,250 µl XL Graduated TipOne[®]])
4. Serological pipettes (Corning, catalog numbers: 4101 [10 ml StripetteTM]; 4051 [5 ml StripetteTM]; 4251 [25 ml StripetteTM])
5. Opti-MEMTM (Thermo Scientific, GibcoTM, catalog number: 11058021, storage conditions: 4°C, shelf life: 12 months)
6. Disposable weighing boats 85 × 85 × 24 mm, PS, medium, white, anti-static (VWR, catalog number: 10770-448, storage conditions: room temperature)
7. 7 ml polycarbonate polypropylene screw cap bijous (container for storage of small-volume samples) (STARLAB, catalog number: E1412-0710)
8. 96-well Delta-treated (hydrophilic surface that promotes cell attachment and growth) White flat-bottom plate (Fisher Scientific, Nunc, MicroWell, catalog number: 10182831)
9. Tissue culture flasks (Greiner Bio-One, catalog numbers: 660160 [175 cm²], 658170 [75cm²])
10. 1.5 ml Microcentrifuge sterile Eppendorf tubes (STARLAB, TubeOne[®], catalog number: S1615-5510)
11. Millex-GP syringe filter unit, 0.22 µm filter, polythersulfone, 33 mm, gamma sterilised (Merck, Millipore, catalog number: SLGP033RS, storage conditions: room temperature)

12. Human Embryonic Kidney 293T, HEK293T cells (ATCC®, catalog number: CRL-3216™, storage conditions: liquid nitrogen vapour phase)
13. Baby Hamster Kidney-21, BHK-21 cells (ATCC®, catalog number: CCL-10™, storage conditions: liquid nitrogen vapour phase)
14. Plasmid DNA: ACE2 receptors (pDISPLAY expression vector, codon-optimised, N-terminal signal peptide [the murine Ig κ-chain leader sequence], C-terminal HA-tag), SARS-CoV Spike, SARS-CoV-2 Spike (pcDNA3.1(+), codon-optimised, C-terminal FLAG-tag) (BioBasic, Canada [Conceicao et al., 2020]), p8.91, CSFLW, VSV-G (pcDNA3.1(+) expression vector) (available upon request), pcDNA3.1(+) (Thermo Scientific, Invitrogen, catalog number: V79020) and pDISPLAY™ (Thermo Scientific, Invitrogen™, catalog number: V66020)
15. Sera or antibodies for neutralisation assays, with relevant biological risk assessment and ethical approvals in place
16. Dulbecco's modified Eagle's medium, DMEM (Merck, Sigma-Aldrich, catalog numbers: D5796 [with phenol red]; D1145 [phenol red free], storage conditions: 4°C, 12 months)
17. Foetal bovine serum, FBS (Life Science Production, catalog number: S-001A-BR, -20°C)
18. Penicillin-Streptomycin, 10,000 U/ml (Thermo Scientific, Gibco™, catalog number: 15240122, storage conditions: -20°C, shelf life: 12 months)
19. Sodium pyruvate, 100 mM (Thermo Scientific, Gibco™, catalog number: 11360070, storage conditions: 4°C, shelf life: 12 months)
20. EDTA (0.5 M), pH 8.0, RNase-free (Thermo Scientific, Ambion®, catalog number: AM9269G)
21. 1× Trypsin-EDTA, 0.25%, phenol red (Thermo Scientific, Gibco™, catalog number: 2520072, storage conditions: -20°C long-term, 4°C while in use, shelf life: 24 months)
22. *TransIT-X2*® Dynamic Delivery System (Mirus, catalog number: MIR 6000, storage conditions: -20°C, shelf life: 12 months)
23. Polyethyleneimine, PEI (Merck, Sigma-Aldrich, catalog number: 408727, storage conditions: 4°C)
24. Nuclease-free, autoclaved, 0.2 µm filtered DEPC-treated water (Ambion, catalog number: AM9906, storage conditions: room temperature)
25. Hydrochloric acid 36.5-38.0%, Bioreagent, for molecular biology (Sigma-Aldrich, catalog number: H1758-100 ml, storage conditions: room temperature)
26. Bright-Glo™ Luciferase Assay System (Promega, catalog number: E2650, storage conditions: -20°C)
27. 55 ml StarTub PVC reagent reservoirs (STARLAB, sterile individually wrapped, catalog number: E2310-1010)
28. DMEM-10% (see Recipes)
29. Working solution of 1 mg/ml PEI (see Recipes)

Equipment

1. Microbiological safety cabinet, BSL-2 (CAS, Biomat 2 – class 2 complies with BS EN 12469:2000)
2. CO₂ incubator (PHC Europe B.V., PHCbi, catalog number: MCO-170AICD-PE)
3. -86°C ultra-low temperature freezer (PHCbi, Panasonic, vip plus, model: MDF-DU900V)
4. -20°C Medical freezer with 14 storage drawers (Liebherr, Profiline, model: G5216)
5. 4°C refrigerator (VDW CoolSystems, Labcold, Sparkfree, model: RLV0217)
6. Sub aqua 5 plus water bath (Fisher Scientific, Grant, model: 13251183)
7. Automated pipettor for serological pipettes (Fisher Scientific, Thermo Scientific™, S1 Pipet Fillers, catalog number: 10072332)
8. Single-channel pipettes (Gilson, Pipetman L, catalog numbers: FA1001M [P2L 0.2-2 µl], FA1003M [P20L 2-20 µl]; FA1005M [P200L 20-200 µl]; FA1006M [P1000L 100-1,000 µl])
9. Multi-channel pipettes (Thermo Scientific™, Finnpipette™ F2 multichannel pipette, catalog numbers: 4662010 [8-well 5-50 µl]; 4662070 [12-well 30-300 µl])
10. Inverted microscope for cell culture (Leica microsystems, model: DMi1-S 40/0.45)
11. Haemocytometer (Fisher Scientific, Hirschmann™ Bright Lined Counting Chambers, catalog number: 105289616)
12. Centrifuge machine (Kendo laboratory product, Sorvall Legend RT, EASYset, model: 75004373)
13. Benchtop autoclave (Fisher Scientific, Astell scientific, catalog number: 12755375)
14. GloMax® Discover Microplate Reader (Promega, catalog number: GM3000)

Software

1. Microsoft Excel (Microsoft 365 for Windows, www.microsoft.com)
2. GraphPad Prism (Version 8.4.2, GraphPad Software for Windows, San Diego, California USA, www.graphpad.com)
3. GloMax® Discover System Software (Version 3.2.3, Promega, Southampton, United Kingdom www.promega.co.uk)

Procedure

- A. Generation of SARS-CoV-2 and SARS-CoV pseudotyped virus particles
1. Maintain HEK293T cells for pseudoparticle production in 25 ml of DMEM-10% (see Recipes) in a 75 cm² tissue culture flask.
 2. Seed HEK293T cells at a concentration of 7.5×10^5 cells per well in a 6-well plate in 3 ml of DMEM-10%, for the total number of wells required.
 3. Agitate cells in the plate using a rapid up-down, left-right movement. This will ensure cells are evenly distributed and do not clump. Incubate at 37°C, 5% CO₂ overnight.
 4. The next day, set up transfection mixes in the afternoon. The seeded HEK293T cells should be between 60-80% confluent for optimal transfection efficiency. Set up transfections for the SARS-

CoV-2 S or SARS-CoV S plasmid, alongside an empty vector negative control (no glycoprotein, no GP) and a positive control. For instance, if the SARS-CoV-2 S and SARS-CoV S plasmids are in a pcDNA3.1 backbone, use an empty pcDNA3.1 plasmid as your no GP control. Generally, a VSV-G plasmid is used as a positive control, as it is trans-encapsidated into the HIV-1 particle efficiently (*i.e.*, it pseudotypes well).

5. In a sterile 1.5 ml Eppendorf tube, add 100 µl of Opti-MEM along with 0.6 µg of p8.91 plasmid (encoding for HIV-1 gag-pol), 0.6 µg of CSFLW plasmid (lentivirus backbone expressing Firefly luciferase), and 0.5 µg of glycoprotein (SARS-CoV-2 S, SARS-CoV S or VSV-G) or empty vector (pcDNA3.1) per well. Incubate for 5 min at room temperature.
6. In a separate 1.5 ml Eppendorf tube, add 100 µl of Opti-MEM plus and 10 µl of PEI (1 µg/ml) per transfection and incubate for 5 min at room temperature.
7. For each 100 µl transfection mix of DNA in Opti-MEM, add 100 µl of PEI in Opti-MEM and mix vigorously with a pipette ten times. Incubate at room temperature for 20 min.
8. Add 200 µl of the volume of the transfection mix in a dropwise manner to each well of the 6-well plate and incubate overnight at 37°C, 5% CO₂.
9. The next morning, use a serological pipette to gently remove the media from wells containing the transfection mix by tilting the dish towards you and aspirating from the edge of the well, being careful not to disturb the monolayer. Replace with 3 ml of DMEM-10%. Incubate overnight at 37°C, 5% CO₂ for 24 h.
10. Harvest cell supernatants containing pseudotyped virus particles and transfer to a 50 ml Falcon, pooling similarly transfected wells, and store at 4°C. Replace the media with 3 ml of DMEM-10% per well, and incubate at 37°C, 5% CO₂ for 24 h.
11. Harvest the cell supernatants containing pseudotyped virus particles and pool with pseudoparticles harvested the day before. Centrifuge at 2,500 × g for 10 min at 4°C to remove cellular debris.
12. Aliquot 4-5 ml of pseudoparticles into bijous and freeze at -80°C until further use.

NB: Larger volume of pseudoparticles can also be prepared in 10 cm² culture dishes. The necessary cell seeding density, DNA concentrations and volumes required for this setup can be found in Table 1, with the corresponding values for the 6-well plate format noted alongside. Steps A9-A12 remain the same regardless of the dish size used, changing only the volume of media required.

Table 1. Quick-guide to generating lentiviral-based pseudotyped viruses

		6-well plate format	10 cm² dish format
Cell seeding density	HEK293T cells	7.5×10^5 per well in 3 ml total volume DMEM-10%	2×10^6 per culture dish in 10 ml total volume DMEM-10%
DNA mix	<i>Viral glycoprotein:</i> SARS-CoV-2/SARS-CoV Spike OR pcDNA3.1 empty vector (NE, negative control) OR VSV-G (positive control)	0.9 µg	1 µg
	p8.91 (HIV-1 gag/pol)	0.6 µg	1 µg
	pCSFLW (HIV-1 LTR, Firefly luciferase gene)	0.6 µg	1.5 µg
	Opti-MEM	100 µl	200 µl
Transfection reagent mix	PEI (1 µg/ml)	10 µl	20 µl
	Opti-MEM	100 µl	200 µl
Total volume per well	Opti-DNA + Opti-PEI mix	200 µl	400 µl
Media	Volume of DMEM-10% to replace after transfection or harvest	3 ml	10 ml

B. Testing SARS-CoV-2 and SARS-CoV pseudoparticle infectivity

1. Seed HEK293T cells at a density of 7.5×10^5 per well in a 6-well plate in a total of 3 ml of DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
2. Ensure plated cells are at 60-80% confluence to ensure optimal transfection efficiency. Set up transfection mixes to test pre-generated SARS-CoV-2 pseudoparticles. In a sterile 1.5 ml Eppendorf tube, add 200 µl of Opti-MEM along with 500 ng of human ACE2 plasmid per well to be transfected. Bring the Tran/T-X2 transfection reagent to room temperature before use, add 2 µl (for every 1 µg of DNA) directly to the tube, and gently flick the tube to mix. Incubate at room temperature for 20 min.
3. Add 200 µl of the transfection mix dropwise to each well of the pre-plated cells and incubate overnight at 37°C, 5% CO₂.
4. Remove the media containing the transfection mix from the wells by tilting the dish towards you and aspirating from the edge of the well using a serological pipette, being careful not to disturb the monolayer. Add 1 ml of DMEM-10% per well and harvest the transfected cells. HEK293T cells have low adherence and come off the plate easily. As such, using the force of the pipetted liquid is sufficient to harvest cells, although care should be taken to ensure a single cell suspension is achieved without clumps. Trypsin should be avoided as this will unnecessarily cleave off the receptors, hampering future experimentation. Transfer to a 50 ml Falcon and dilute cells to 2×10^5 /ml with DMEM-10%.
5. Seed 100 µl of diluted cells (2×10^4 per well) into a flat, white-bottomed 96-well plate and incubate overnight at 37°C, 5% CO₂.

6. The next day, thaw an aliquot of SARS-CoV-2 and/or SARS-CoV pseudoparticles, along with the negative (pcDNA3.1, no GP) and positive (VSV-G) controls. Titrate the pseudoparticles in a clear-bottomed 96-well plate, starting with undiluted virus in the top row, and titrating 10-fold in DMEM-10%, for a final volume of 100 µl.
 7. Gently remove the media from the white-plate seeded with human ACE2-transfected cells and add 100 µl titrated pseudoparticles. Incubate for 48 h at 37°C, 5% CO₂.
 8. Remove the media from the wells by tilting the dish towards you and aspirating from the edge of the well using a multi-channel pipette and add 50 µl Bright-Glo™ diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min and then measure the luciferase signal on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.
 9. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.
- C. ACE2 receptor usage screen using SARS-CoV-2 and SARS-CoV pseudotyped virus particles (Conceicao *et al.*, 2020)
1. Maintain BHK-21 cells in 25 ml DMEM-10% in a 75 cm² tissue culture flask. Seed BHK-21 cells in 24-well plates at 1×10^5 /well in DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
 2. Ensure plated cells are at 60-80% confluency to ensure optimal transfection efficiency. Set up transfection mixes in 100 µl of Opti-MEM along with 500ng of different species of ACE2-expressing constructs or an empty vector control (e.g., pDISPLAY). Bring the Tran/T-X2 transfection reagent to room temperature before use and add 3 µl for every 1 µg of DNA directly to the tube and gently flick the tube to mix. Incubate at room temperature for 20 min.
 3. Add 100 µl of the transfection mix dropwise to each well of the pre-plated BHK-21 cells and incubate overnight at 37°C, 5% CO₂.
 4. Remove the media containing the transfection mix from the wells and add 0.5 ml of 2 mM EDTA in PBS per well to harvest the transfected cells. Transfer to a bijou and dilute cells to 2×10^5 /ml with DMEM-10%.
 5. Seed 100 µl of diluted cells (2×10^4 per well) into a flat, white-bottomed 96-well plate and incubate overnight at 37°C, 5% CO₂.
 6. Remove media from cells and infect with SARS-CoV-2 or SARS-CoV pseudoparticles equivalent to 10^6 - 10^7 relative light units (RLU), or the no GP control at the same dilution and incubate for 48 h at 37°C, 5% CO₂.
 7. Remove the media from the wells and add 50 µl of Bright-Glo™ diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min then read on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.
 8. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.

D. Neutralisation assay using SARS-CoV-2 and SARS-CoV pseudotyped virus particles

1. Prior to setting up neutralization assays, seed HEK293T cells at a density of 7.5×10^5 per well in a 6-well plate in a total of 3 ml of DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
2. Ensure plated cells are at 60-80% confluence to ensure optimal transfection efficiency. In a sterile 1.5 ml Eppendorf tube, add 200 µl of Opti-MEM along with 500 ng of human ACE2 plasmid per well to be transfected. Bring the Tran/T-X2 transfection reagent to room temperature before use and add 2 µl for every 1 µg of DNA directly to the tube and gently flick the tube to mix. Incubate at room temperature for 20 min.
3. Add 200 µl of the transfection mix dropwise per well of pre-plated cells and incubate overnight at 37°C, 5% CO₂.
4. Set up neutralisation assays by diluting sera/monoclonal antibodies (mAbs)/inhibitors considering the dilution series to be used and the final volume after addition of pseudoparticles. For example, sera to be titrated using a 2-fold dilution series starting at a 1:10 dilution would require 10 µl sera per well in 100 µl serum free DMEM. The same is applicable for mAbs or inhibitors with a known concentration.
5. Add 100 µl of diluted sera/mAbs/inhibitors in triplicate to the top row of a flat white-bottomed 96-well plate. Add 50 µl of serum free media to all remaining wells. Remove 50 µl from the top row and titrate 2-fold down the plate, mixing well before each titration. Do not titrate into the bottom row. This whole row will be used as the untreated control.
6. Thaw an aliquot of SARS-CoV or SARS-CoV-2 pseudoparticles and dilute in serum free DMEM, equivalent to ~10⁶ RLU and add 50 µl per well, including the untreated controls. Incubate for 1 h at 37°C, 5% CO₂.
7. Remove the media from the 6-well plates transfected with human ACE2. Add 1 ml of DMEM-10% per well and harvest the transfected cells. HEK293T cells have low adherence, so come off the plate easily; therefore, the force of the pipetted liquid should be sufficient to harvest cells (see B4 above). Transfer to a 50 ml Falcon and dilute cells to 2×10^5 /ml with DMEM-10%.
8. Seed 100 µl of diluted cells (2×10^4 per well) onto each well containing sera/mAb/inhibitor with pseudoparticles and the untreated controls. Incubate for 48 h at 37°C, 5% CO₂.
9. Remove the media from the wells and add 50 µl of Bright-Glo™ diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min then read on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.
10. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.

Data analysis

A. Testing SARS-CoV-2 and SARS-CoV pseudoparticle infectivity

1. After preparing a batch of pseudoparticles, their infectivity can be tested by titrating them on target cells that have been transfected to express the host receptor (ACE2) of the pseudotyped

attachment protein (Spike) for SARS-CoV and SARS-CoV-2. Undiluted pseudotyped virus (“1”) is titrated 10-fold with DMEM-10% down a 96-well plate in triplicate (“10”, “100”, “1,000” etc.) (**Figure 1A**).

2. Measuring the luciferase signal of the pseudoparticles will generate a CSV file that can be exported onto a USB flash drive and analysed on Microsoft Excel. These results can then be plotted on GraphPad Prism to show the mean \pm SD for each pseudoparticle.
3. The no GP negative control serves as an indication of background luciferase signal, and only values above this at each corresponding dilution should be considered as a true luciferase signal for the pseudoparticles being tested (**Figure 1B, black line**). Generally, a minimum of \sim 2 log dynamic range between the no GP and the pseudotyped virus and a RLU signal between $10^{5.5}$ and $10^{7.5}$ RLU (**Figure 1B, shaded area**) is sufficient for use in subsequent assays. The titration series will also help to determine the lowest usable dilution of the pseudoparticles to still obtain meaningful luciferase values above the background. Of note: the following data to be discussed was generated for illustrative purposes only.
4. For example, when considering the luciferase values obtained for SARS-CoV, although these are above the no GP control at the highest dilution, the difference between the two is only \sim 1 log, which falls outside our criteria for use (**Figure 1B, blue line**). When titrating the pseudoparticles, the luciferase values also fall quite quickly below the lower limit of the workable range at a 1:10 dilution and to the same values as the no GP control by the 1:1000 dilution, making the titre of this preparation of SARS-CoV pseudoparticles unsuitable for use in subsequent assays (**Figure 1B**).
5. In comparison, the luciferase signals obtained for SARS-CoV-2 are \sim 2 log above the background no GP control, and a dilution of 1:10 of the pseudoparticles would be within the workable range for use in subsequent assays, which is lost at a 1:100 dilution (**Figure 1B, orange line**).
6. The VSV-G pseudoparticles are a positive control within the assay, where the luciferase values observed should be above 10^7 RLU (**Figure 1B, green line**).

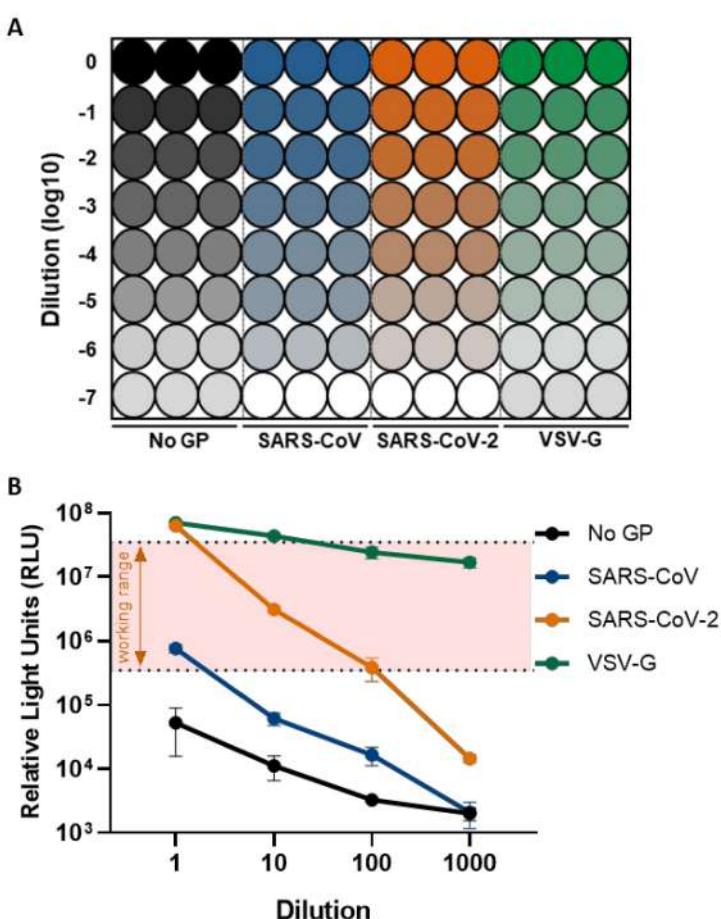


Figure 1. Testing the infectivity of SARS-CoV and SARS-CoV-2 pseudoparticles. (A) For SARS-CoV and SARS-CoV-2 pseudoparticle titrations, 10-fold serial dilutions of the supernatant are used to infect HEK293T cells transiently expressing the human ACE2 receptor in a white flat-bottomed 96 well-plate. Negative (no GP) and positive (VSV-G) controls are also included in the experiment. Each condition is tested in triplicate. (B) Two days after infection, signal luciferase values are measured and plotted as the mean \pm SD. The no GP control is indicative of the background, and only pseudoparticle values above this should be considered as true infectivity, matched at each dilution of the virus. The use of pseudoparticles for subsequent neutralisation assays and receptor usage screens should show \sim 2 log dynamic range between the no GP and pseudotyped virus and fall between a working range of $10^{5.5}$ to $10^{7.5}$ (shaded area).

- B. ACE2 receptor usage screen using SARS-CoV-2 and SARS-CoV pseudotyped virus particles
- Infection of cells expressing various species ACE2 receptors with SARS-CoV or SARS-CoV-2 pseudoparticles set up in triplicate yields luciferase signals that can be plotted alongside each other (mean \pm SD) to depict raw values. These data also give an idea of the general trend of receptor tropism across different viruses (**Figure 2A**).
 - For example, water buffalo and goat ACE2 permit the entry of SARS-CoV and SARS-CoV-2

pseudoparticles well, which is less evident for little brown bat ACE2. Differences between viruses can be observed for civet ACE2, which permits the entry of SARS-CoV more efficiently than SARS-CoV-2 (**Figure 2A**). These experiments should be conducted at least three times on three separate occasions, with representative data shown. A subset of ACE2 receptors are shown in **Figure 2A**, but a more in-depth, wider analysis can be found in Supplementary Figure 3A and 3C in Conceicao *et al.* (2020).

3. Two negative controls are set up in this screen. The first is an empty vector control (pDISPLAY) to ensure any signal measured is solely from overexpression of the ACE2 receptor. The second is infection of cells with the no GP control pseudoparticle preparation to ensure luciferase signals can be attributed to the pseudotyped viruses and provides a baseline for the background (**Figure 2A**).
4. The raw luciferase signals can then be used to determine the relative usage of non-cognate host ACE2 receptors (water buffalo, civet, goat, little brown bat) to a known or cognate host receptor, in this case, human ACE2. The mean percentage from three separate experiments performed on different days are used to obtain these values. The luciferase value for human ACE2 is set to 100%, and the luciferase values for unknown host receptors and the negative control are then expressed as a percentage relative to human ACE2.
5. These results can also be shown as a heatmap using a colour gradient to show different trends of receptor usage. For example, human ACE2 (100%) is set as green. Expression lower than this is shaded from green to red, indicative of poorer ACE2 usage relative to human ACE2. Values above 100% are shown as a darker green, suggestive of ACE2 usage equivalent to or greater than human ACE2 (**Figure 2B**). A subset of ACE2 receptors are shown in **Figure 2B**, but a more in-depth, wider analysis can be found in Figure 2 A in Conceicao *et al.* (2020).
6. Further analysis can be carried out to compare the receptor tropism of different species ACE2 between SARS-CoV and SARS-CoV-2 by plotting the percentage values for each virus against each other on an xy scatter graph, calculating the Pearson's correlation coefficient, and plotting a linear line of regression fitted with 95% confidence intervals (data not shown). An example of such analysis can also be found in Supplementary Figure 5 in Conceicao *et al.* (2020).

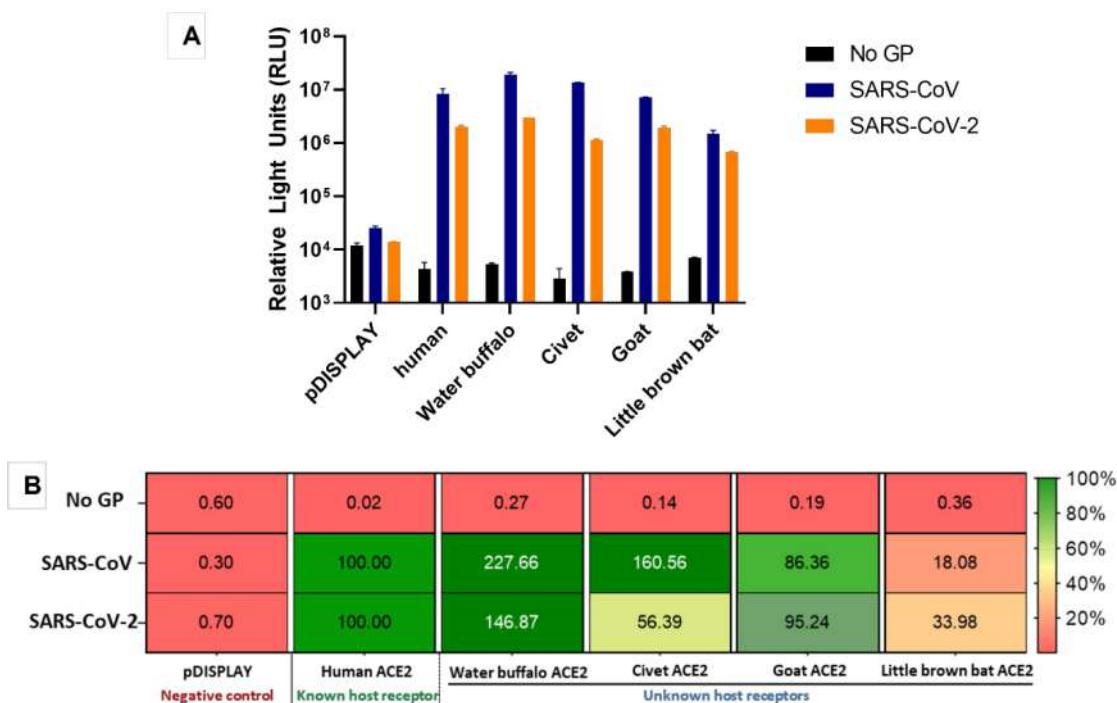


Figure 2. ACE2 receptor usage screen using SARS-CoV and SARS-CoV-2 pseudoparticles. In our study, pseudoparticles were used as a surrogate to live viruses to assess receptor tropism of SARS-CoV and SARS-CoV-2 with different species of ACE2 receptors. Pseudoparticles were employed to assess SARS-CoV and SARS-CoV-2 Spike glycoproteins' usage of ACE2 receptors from different species and presented as **(A)** raw luciferase signal values or **(B)** a percentage relative to human ACE2. Negative controls were included for pseudoparticles bearing an empty vector control (No GP) or mock-transfected with an empty vector in place of an ACE2 receptor (pDISPLAY). Data are presented as mean \pm SD of triplicate values, with each experiment performed three times on three separate occasions, and representative data shown.

C. Neutralisation assay using SARS-CoV-2 and SARS-CoV pseudotyped virus particles

- Neutralisation assays using pseudotyped viruses are a low-biocontainment alternative to using live virus and can be performed in a relatively high-throughput manner. These neutralisation assays can be performed on mAbs, sera or any other drug or inhibitor that has the potential to inhibit viral entry. The data discussed herein have been generated for illustrative purposes only.
- Inhibitors (mAbs/sera/drugs) of a known concentration can be titrated down a 96-well plate in triplicate to determine the extent of inhibition of SARS-CoV-2 entry. This is done by taking an average of the untreated controls and expressing the RLU values for each individual replicate of the mAb of interest relative to this. These can then be plotted as the mean \pm SD and should be repeated a minimum of three times, with representative data shown:

$$(\text{RLU individual replicate of mAb}/\text{RLU average of untreated}) \times 100$$

3. The inhibitory concentration of 50% (IC₅₀) should be indicated on a graph along with the untreated, no mAb control (100%). Values below this IC₅₀ line are indicative of S-mediated inhibition of entry, which can be calculated at each concentration. For example, mAb2 is able to inhibit SARS-CoV-2 S entry by ~80% (20% of untreated) at 100 µg/ml. The values obtained for mAb2 at all concentrations tested are below the IC₅₀ value, so lower concentrations would need to be tested to determine the limit of inhibition. In contrast, mAb3 inhibits SARS-CoV-2 S entry by ~90% at 100 µg/ml, but at 12.5 µg/ml, the inhibition is now above the IC₅₀ threshold (**Figure 3A**).
4. There may also be examples of mAbs that do not inhibit SARS-CoV-2 S, as with mAb1. It may be possible that when inhibition of entry is not observed, a slight increase above the 100% threshold is seen. The mechanisms causing this increase are still unknown and under investigation, but for the purposes of this assay, the conclusion that the mAb does not neutralise SARS-CoV-2 S is sufficient (**Figure 3A**). Examples of this sort of analysis can be found in Thakur et al. (2021).
5. When determining the inhibition of viral entry from individuals who have antibodies against SARS-CoV-2 S, whether that be following natural infection or vaccination, a neutralising antibody titre is usually calculated to enumerate the level of SARS-CoV-2 S neutralisation. The simplest method of calculating this is by calculating the average RLU of the untreated controls and determining the IC₅₀ value, i.e., 50% of the no sera control. The neutralisation titre is then calculated as the inverse of the dilution at which there is 50% inhibition of the no sera luciferase values in all triplicate wells. These titres can then be tabulated or plotted on a log scale.
6. For example, the titre of 256 for serum sample 1 and 512 for serum sample 3 indicates that IC₅₀ was calculated at a dilution of 1:256 and 1:512, respectively. The conclusion that could be drawn from this is that serum sample 3 is able to neutralise SARS-CoV-2 S-mediated entry more efficiently than serum sample 1 and therefore has higher antibody titres (**Figure 3B**).
7. For serum sample 4, this value has been plotted as 1,024, which is the upper limit of detection (ULoD) for this assay. This means that this sample was able to inhibit 50% of the luciferase signal in all wells and at the lowest dilution that was tested. This serum would have to be retitrated with a broader dilution series to determine the neutralisation titre. For serum sample 2, none of the wells in the dilution range yielded a recordable IC₅₀. The neutralisation titre is therefore plotted as an arbitrary value below the lower limit of detection (LLoD), which in this case is 40, but would be reported as <40 as the true titre is unknown (**Figure 3B**). Examples of IC₅₀ neutralisation titres using this method can be found in Figure 4F in Thakur et al. (2021) and in Figure 2D and 2E in Graham et al. (2020).

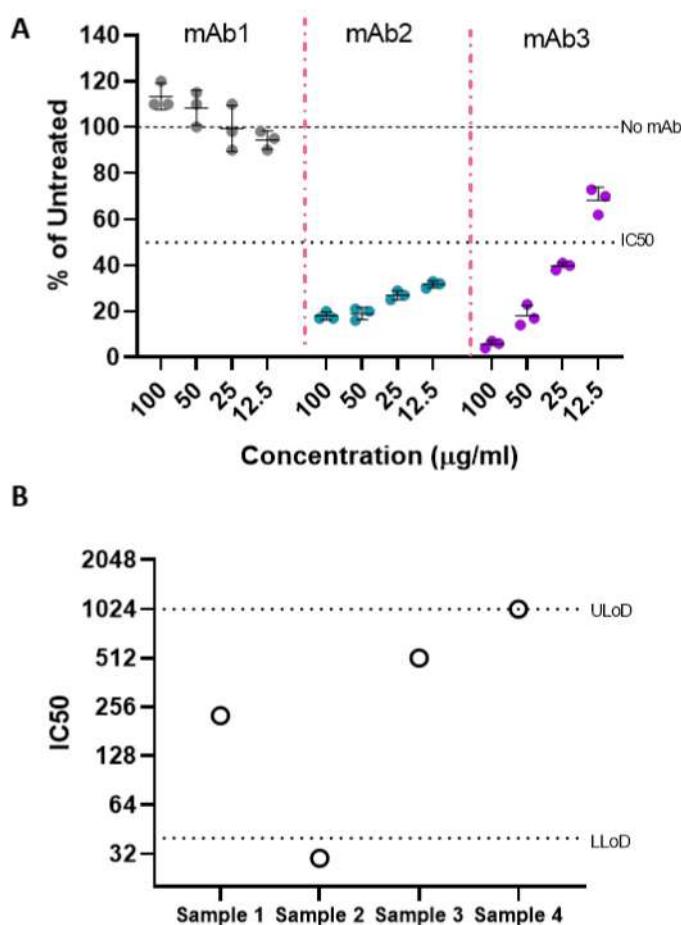


Figure 3. Neutralisation assays using SARS-CoV-2 pseudoparticles. SARS-CoV-2 Spike neutralisation assays were performed in the presence of (A) monoclonal antibodies (mAbs), presented as a percentage relative to untreated controls or (B) sera samples, with data expressed as neutralising titres. SARS-CoV-2 pseudoparticles were incubated with mAbs or sera for 1 h prior to addition of human ACE2-expressing HEK293T cells. Inhibition of SARS-CoV-2 Spike-mediated viral entry was determined by calculating the concentration (mAbs) or dilution (sera) at which there is a 50% reduction in luciferase signal (IC₅₀). Data represent the mean \pm SD of triplicate values, with each experiment performed three times on three separate occasions, and representative data shown.

Notes

1. Procedure A, step 11 mentions centrifugation of pseudoparticle preparations prior to use in subsequent assays to remove cellular debris. Other protocols require further filtration of pseudoparticles using a 0.45 μm filter before storage. This step is not carried out in our lab, as we have observed a reduction in infectivity following filtration.
2. Manufacturers usually recommend an optimal confluence of 60-80% for transfection. Therefore, it may be necessary to change the seeding density depending on the characteristics of the cells

used. For example, if the cell types used are larger (e.g., BHK-21 cells are larger than HEK293T cells) or have a high doubling rate, we recommend starting at a lower seeding density. On the other hand, if the cells are smaller, or have a slower growth rate, and are difficult to reach confluence (e.g., Calu3 cells) or indeed are suspension cells, you may want to start with a higher seeding density. In both instances, we recommend testing different seeding densities to find the optimal for any given experiment.

3. An example of optimal confluence of HEK293T cells prior to infection (60-80%) is shown in **Figure 4**. Cells should be evenly distributed across the well (*i.e.*, no clumping or aggregation in one area), with visible gaps in the monolayer (**Figure 4A**). The cell morphology of the HEK293T cells should appear flat and polygonal at confluence, which indicates adherence to the plastic (**Figure 4B**).

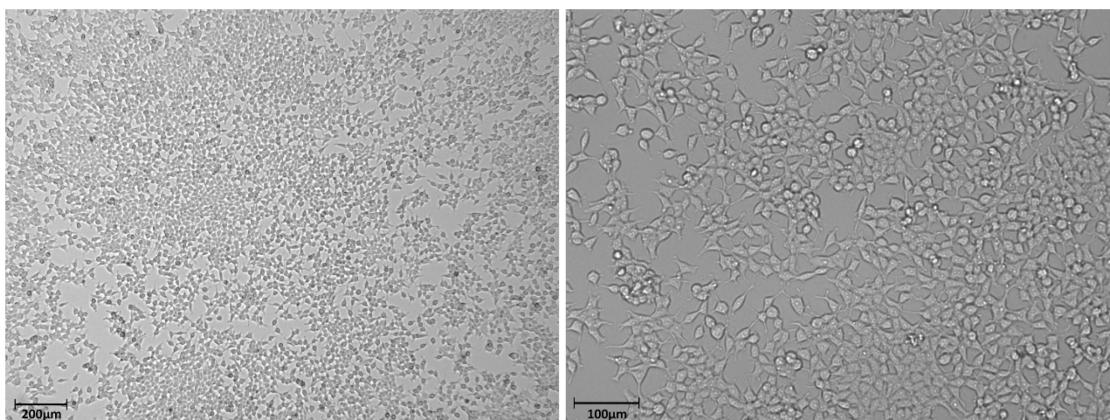


Figure 4. Brightfield image to illustrate 60-80% optimal confluence of HEK293T cells.

HEK293T cells were seeded at 7.5×10^5 in a 6-well dish in 3ml of DMEM-10%. On the following day, cells should be between 60-80% confluent for optimal transfection. **(A)** Example of ~80% confluent HEK293T at 4 \times magnification, with cells appearing evenly distributed across the well and visible gaps. **(B)** Higher power magnification (10 \times) of HEK293T cells where cells should appear bright, flat and polygonal prior to transfection.

4. The concentrations of DNA used for transfection at various steps have been optimised for use in these assays. It is important that any plasmids used are optimised to account for variability in vector platform and codon-optimisation.
5. A higher signal (in the 10^4 - 10^5 RLU range) in the no GP control can sometimes be seen, likely the result of non-specific uptake of ‘bald’ pseudoparticles or debris from producer cells. To reduce this background signal, care should be taken to ensure producer cells are not transfected at low confluence as this can cause cytopathic effects (CPE) to develop.
6. All the experiments conducted here were performed with over-expressed ACE2 only. Co-expression of the serine protease TMPRSS2, which is required for S2 protein cleavage to S2', can facilitate the fusion of viral and cellular membranes and cleavage of the Spike protein (Hoffmann *et al.*, 2020). TMPRSS2 was not included in our host range assays as we wanted to

specifically examine the effects of different ACE2s – indeed, over-expression of TMPRSS2 led to ACE2 restrictions being masked (Conceicao *et al.*, 2020).

7. Other formulae can be used to determine the IC₅₀ value, yielding different titres. This is acceptable if the same method is used throughout analyses and the method used are described in full. Other formulae for calculating neutralisation titres include (1) using a non-linear regression analysis tool on GraphPad Prism after plotting data on an XY graph to interpolate neutralisation values (Ferrara and Temperton, 2018), (2) interpolating the point at which infectivity is reduced to 50% of the value of a no serum control sample using a fixed formula (Logan *et al.*, 2016), and (3) determining the highest dilution at which complete neutralisation is seen in all replicate wells and considering other wells that also show neutralisation. Neutralisation is then calculated by inputting these values into a Spearman Karber formula (Lambe *et al.*, 2021).
8. Neutralisation titres do not always need to be recorded as IC₅₀ values. Other cut-off points can be chosen dependent on the level of neutralisation expected in a given assay, and to provide a more stringent measure of neutralisation (e.g., 80% neutralisation, IC₈₀).
9. The surface expression of different ACE2 receptors may differ, which may affect the level of Spike-ACE2 interaction leading to misinterpretation of results. Therefore, it is important to investigate and normalise the cell surface expression of the ACE2 receptors used. The mammalian ACE2 receptors described and used herein were HA-tagged at the C-terminus, which allowed detection of surface expression by flow cytometry. Additionally, protein expression was assessed by Western blotting (Conceicao *et al.*, 2020).

Recipes

1. DMEM-10%

DMEM supplemented with 10% FBS, 1% penicillin/streptomycin 10,000 U/ml, and 1% 100 mM sodium pyruvate, cultured at 37°C with 5% CO₂.

2. Working solution of 1 mg/ml PEI

- a. Weigh the viscous liquid to get 50 mg/ml in water (e.g., 0.42 g PEI + 8.4 ml water) and transfer to a sterile 50 ml Falcon.
- b. Place Falcon in a water bath set to 50°C and gently pipette up and down using a 1 ml pipette until fully dissolved.
- c. Dilute to 1 mg/ml with water (e.g., take 0.5 ml of your 50 mg/ml stock and add 24.5 ml water)
- d. The solution in its current state will be very basic. Adjust pH to 7 using diluted hydrochloric acid.
- e. Filter through a 0.22 µm filter and aliquot into 1.5 ml Eppendorf tubes.
- f. Store at -20°C long-term and at 4°C for up to one month while in use.

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Competing interests

Authors declare no conflicts of interest.

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Production, Titration, Neutralisation, Storage and Lyophilisation of Severe Acute Respiratory

Syndrome Coronavirus 2 (SARS-CoV-2) Lentiviral Pseudotypes

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[Abstract] This protocol details a rapid and reliable method for the production and titration of high-titre viral pseudotype particles with the SARS-CoV-2 spike protein (and D614G or other variants of concern, VOC) on a lentiviral vector core, and use for neutralisation assays in target cells expressing angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). It additionally provides detailed instructions on substituting in new spike variants via gene cloning, lyophilisation and storage/shipping considerations for wide deployment potential. Results obtained with this protocol show that SARS-CoV-2 pseudotypes can be produced at equivalent titres to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) pseudotypes, neutralised by human convalescent plasma and monoclonal antibodies, and stored at a range of laboratory temperatures and lyophilised for distribution and subsequent application.

Keywords: SARS-CoV-2 coronavirus, Lentiviral pseudotype, Virus neutralisation, Spike variants, Pseudotype lyophilisation, COVID-19

[Background] SARS-CoV-2 is the causative agent of COVID-19 disease currently manifesting as a global pandemic (Zhu *et al.*, 2020). Due to the highly infectious nature of SARS-CoV-2, the wild-type virus has been classified as a BSL-3 pathogen, heavily restricting its use in many laboratories. To circumvent this biohazard restriction, pseudotype viruses (PVs) can be generated by utilising a surrogate viral core to generate virions displaying the SARS-CoV-2 spike protein (Nie *et al.*, 2020; Crawford *et al.*, 2020). Due to the single-round infection and replication deficient properties of PVs, they can be employed in BSL-2 laboratories. The use of PVs as a platform to investigate serosurveillance, antigenic properties and viral entry mechanisms of emerging viruses has been extensively reviewed (Bentley *et*

al., 2015; Li et al., 2018; Cantoni et al., 2021; Focosi et al., 2021), with many studies demonstrating a high degree of correlation between wild-type virus neutralisation assays and PV neutralisation assays (Hyseni et al., 2020; Schmidt et al., 2020; Sholukh et al., 2020; Xiong et al., 2020). Furthermore, PVs can be used as a diagnostic control for new platforms to detect SARS-CoV-2 infection in patients (Sholukh et al., 2020).

Since the start of the pandemic, many protocols have been established for generating SARS-CoV-2 pseudotypes using a range of viral cores, all of which have shown that long term storage of PVs is ideal at -80°C (Crawford et al., 2020; Nie et al., 2020). The issue that this presents is that sharing PVs with research or diagnostic laboratories that do not have expertise in PV generation and application is that it would incur high shipping costs, as the particles need to be shipped on dry ice to remain stable. Additionally, many laboratories within low-income and middle-income countries (LMICs) have no routine access to -80 °C storage. In this protocol, we present our method of generating (Figure 1) and using lentiviral based PVs (highly adaptable and biosafe) expressing SARS-CoV-2 spike, with the additional step of lyophilisation of SARS-CoV-2 PVs using sucrose as a cryopreservant and a freeze drier. Our previous experience has shown that lyophilisation of Influenza, Rabies, and Marburg PVs does not affect PV performance (Mather et al., 2014). We show that SARS-CoV-2 PV can be lyophilised in the same manner and can thus be transported at room temperature over extended time periods (at least a week) and reconstituted into solution without any loss of PV performance (for similar PV, reconstitute within a year if kept at 4°C). This will greatly expand the adoption of pseudotype SARS-CoV-2 neutralisation assays globally and facilitate diagnostic platforms that use SARS-CoV-2 PVs as control samples, like MALDI-ToF (Iles et al., 2020), from both a financial and practical point of view.

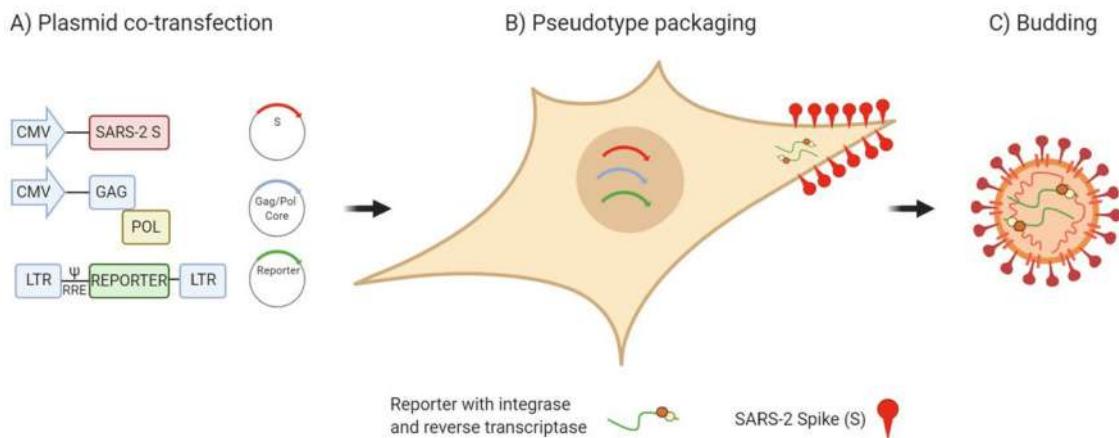


Figure 1. Schematic representation of the production of SARS-CoV-2 pseudotype viruses.
HEK293T/17 cells are transfected with three plasmids (Lentiviral vector incorporating luciferase reporter, packaging construct and SARS-CoV-2 spike expression plasmid) for the production of SARS-CoV-2 Spike bearing lentiviral pseudotypes.

Materials and Reagents

1. MultiGuard Barrier pipette tips 1-20 and 1-200 µl (Sorenson BioScience, catalog number: 30550T)
2. Nunc™ Cell Culture Treated Multidishes (6-well) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 140675)
3. Nunc™ Cell Culture Dish Delta Surface Treated (10 cm sterile dishes) (Thermo Fisher Scientific, Thermo Scientific™, catalogue number: 150350)
4. Reaction tube, 1.5 ml with attached cap, graduation and writing area (Greiner Bio-One, catalog number: 616201)
5. Fisherbrand™ Sterile Syringes for Single Use 3 ml, (Fisher Scientific, Thermo Scientific™, catalog number: 14955457)
6. 0.45 µm syringe filter, cellulose acetate (STARLAB, catalog number: E4780-1453)
7. Pipette basins (50 ml), Generic
8. 96-well white plate (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 136101)
9. Microtube (1.5 ml, 0.5 ml), generic
10. Thin-walled PCR microtubes (0.2 ml), generic
11. HEK 293T/17 cells (ATCC, catalog number: CRL-11268)
12. Subcloning efficiency *E. coli* DH5α cells (Invitrogen, catalog number: 18265017)
13. Plasmids
 - a. Spike plasmid: pCAGGS-SARS-CoV spike (CFAR, catalog number: 100976)
 - b. Lentiviral vector expressing firefly luciferase: pCSFLW (or pCSGW for GFP PV) (Carnell et al., 2015)
 - c. Second-generation lentiviral packaging construct plasmid: p8.91 (expresses gag, pol and rev) (Carnell et al., 2015)
 - d. Host cell entry receptor ACE2 expression plasmid: pCDNA3.1+-ACE2 (Simmons et al., 2004)
 - e. Coronavirus Spike (S) protein priming TMPRSS2 expression plasmid: pCAGGS-TMPRSS2 (Bertram et al., 2010)

Note: Information on the plasmids above can be found in Temperton et al. (2005) and Carnell et al. (2015). Plasmids available from VPU.
14. Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L Glucose (Pan-Biotech, catalog number: P04-04510) supplemented with 10% foetal bovine serum (FBS) (Pan-Biotech, catalog number: P40-37500) and 1% penicillin/streptomycin (P/S) (Pan Biotech, catalog number: P06-07100)
15. Gibco Reduced Serum media Opti-MEM® (Thermo Fisher Scientific, catalog number: 51985034)
16. FuGENE® HD Transfection Reagent, 1 ml (Promega, catalog number: E2311)
17. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Pan-Biotech, catalog number: P04-36500)
18. Trypsin-EDTA (0.05%), phenol red (Pan-Biotech, catalog number: P10-040100)

19. Positive control antibody (Research Reagent for Anti-SARS-CoV-2 Ab) that can neutralise the SARS-CoV-2 PV (NIBSC, code: 20/130, available internationally)
20. COVID-19 human convalescent plasma panel (NIBSC, catalog number: 20/118)
21. Monoclonal antibodies that can neutralise the SARS-CoV-2 PV (Native Antigen, catalog numbers: MAB12443 and MAB12444)
22. Bright Glo™ luciferase assay system (Promega, catalog number: E2650)
23. Low surface tension polypropylene 1.5 ml microtubes (Simport, catalog number: T330-7LST)
24. Sucrose (Sigma-Aldrich, catalog number: S0389)
25. Dulbecco's Phosphate Buffered Saline (DPBS; Pan Biotech, catalog number: P04-361000)
26. Spike plasmid: pCAGGS-SARS-CoV spike (CFAR, catalog number: 100976)
27. Eukaryotic expression vector recipient plasmids: pcDNA3.1, pl.18, pCAGGS (Carnell et al., 2015)
28. Anza enzyme kit (Thermo Fisher Scientific, catalog number: IVGN3006)
29. Nuclease-free water (Thermo Fisher Scientific, catalog number: R0582)
30. QIAquick PCR Purification kit (Qiagen, catalog number: 28104)
31. QIAquick Gel Extraction kit (Qiagen, catalog number: 28704)
32. Tris Acetate EDTA (TAE) buffer (50× concentrate; Thermo Fisher Scientific, catalog number: B49)
33. Ultrapure Agarose (Thermo Fisher Scientific, catalog number: 16500100)
34. SYBR Safe DNA gel stain (Invitrogen, catalog number: S33102)
35. GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, catalog number: SM0313)
36. Luria Broth (LB) and LB agar powder (Sigma-Aldrich, catalog numbers: L3022 and L2897)
37. DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, catalog number: K1081)
38. Monarch plasmid miniprep kit (New England Biolabs, catalog number: T1010S) or alternative

Equipment

1. Class II biosafety cabinet (Thermo Fisher Scientific, Thermo Scientific™, model: MSC-Advantage™)
2. Water bath or incubator, generic
3. Pipettes (Gilson, models: PIPETMAN® Classic, P2, P20, P200 and P1000 or equivalent)
4. Multichannel pipette (Gilson, model: PIPETMAN L Multichannel P12 20-200 µl or equivalent)
5. Fisherbrand™ Sterile Polystyrene Disposable Serological Pipets 5 ml and 10 ml in 1/10 ml, Sterile, Plugged, Individually Wrapped (Fisher Scientific, Thermo Scientific™, catalog numbers: 1367610H and 1367610J)
6. Portable Pipet-Aid® XP Pipette Controller (Drummond Scientific Company, catalog number: 4-000-101 or equivalent)
7. Vortex Mixer, adjustable speed (SciQuip, model: SP2260-VM)
8. Galaxy MiniStar Mini Centrifuge (VWR, model: C1413V-230)

9. Optional: BIO-RAD TC20™ Automated Cell Counter (Bio-Rad Laboratories, catalog number: 1450102EDU) or FastRead 102 disposable 10-chamber counting grid with integral acrylic, optically clear, coverslip (Immune Systems, catalog number: BVS100)
10. Plate centrifuge (ELMI, model: SkyLine CM-6MT)
11. GloMax® Navigator Microplate Luminometer (Promega, model: GloMax® Navigator)
12. FreeZone 2.5 L freeze dryer (LabConCo, catalog number: 7670520)
13. Sample drying chamber (LabConCo, catalog number: 7318700)
14. Rotary Vane Vacuum pump 117 (LabConCo, catalog number: 7739402)
15. Plastic microtube rack (Thermo Fisher Scientific, catalog number: 8850)
16. Thermo-humidity meter (Thermo Fisher Scientific, catalog number: 11536973)
17. Water bath or heat block, generic
18. Microwave, generic
19. Powerpack, generic
20. Gel electrophoresis tank, generic
21. Microcentrifuge (Thermo Fisher Scientific, Sorvall™ Legend™)
22. UV transilluminator (Uvitech/Sigma, catalog number: Z363677)
23. Gel imaging system, generic
24. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, catalog number: ND-2000)
25. Dry 37°C incubator, generic
26. Shaking 37°C incubator, generic
27. Thermocycler (Techne, model: Prime, catalog number: 5PRIMEG/02)

Software

1. PC or Mac with Microsoft Excel (Microsoft®)
2. GraphPad Prism® (GraphPad Software)

Procedure

A. Production of SARS-CoV-2 PV via plasmid co-transfection of 293T cells (4-5 days)

Note: All steps should be carried out in a class II biosafety cabinet to avoid contamination.

1. Day 1: 293T/17 cells should be sub-cultured into 6-well plates at a ratio that will deliver 70-90% confluence at the time of transfection (Day 2). Typically seeding 4×10^5 cells per well will achieve this level of confluency. An example of what the cells should look like is shown in Figure 2.

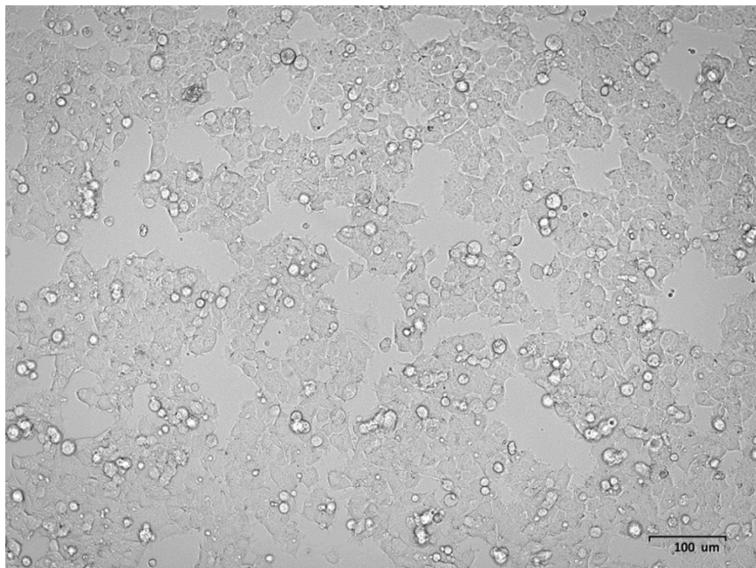


Figure 2. Example of the confluence expected prior to transfection of HEK293T/17 cells with plasmids

2. Day 2: DMEM/10% FBS/1% P/S and Opti-MEM® should be pre-warmed to 37°C using a water bath or an incubator.
3. Prepare two labeled sterile 1.5 ml microcentrifuge tubes (tube 1 and tube 2) for each well of a 6-well plate which will be used for transfections.
4. Add the following plasmid constructs (in a total volume of 15 µl) for transfection to tube 1:
pCAGGS-SARS-CoV-2 or pCAGGS-SARS-CoV-2 (D614G) spike: 450 ng.
p8.91-lentiviral vector: 500 ng.
pCSFLW: 750 ng.
Note: Volume of each individual plasmid determined by its concentration.
5. Add 100 µl Opti-MEM® to the plasmid DNA mix (tube 1).
6. Add 100 µl Opti-MEM® and FuGENEHD to tube 2.
7. Incubation step: Mix both tubes by gently flicking and incubate for 5 min at room temperature (RT).
8. After incubation, pipette the Opti-MEM®/FuGeneHD solution from tube 2 into the Opti-MEM®/DNA solution in tube 1.
9. Incubation step: Incubate the tube at RT for 20 min whilst gently flicking the tube to mix every 3-4 min.
10. Whilst the transfection mix is incubating, the culture media on the 293T/17 cells should be removed and 2 ml of fresh prewarmed DMEM/10% FBS/1% P/S added per well. It is imperative at this point to add culture media slowly to one side of the well to avoid detaching the adherent cell monolayer.
11. After 20 min incubation, pipette the DNA/Opti-MEM®/FuGeneHD solution onto the 293T/17 cells (in one well of a 6-well plate) by adding dropwise throughout the total surface area of the well. Swirl the 6-well plate (s) gently to ensure an even dispersal of reagent mix.

12. Incubation step: Incubate the plate at 37°C, 5% CO₂ for 44-52 h. In our hands, incubation times in this range result in equivalent final PV production relative luminescence unit (RLU) titres.
13. After overnight incubation (on Day 3), the culture media on the cells should be carefully removed and 2 ml fresh DMEM/10% FBS/1% P/S added. Again, add media slowly to one side of the well to avoid cell detachment.
14. Day 4: Supernatant containing the viral pseudotype particles are harvested using a 3 ml sterile syringe and subsequently filtered into microcentrifuge or Falcon tubes via a syringe driven 0.45 µm filter.
15. Store all filtered supernatants at -80°C until downstream use. It is recommended that supernatant is stored as aliquots to avoid multiple freeze-thaw cycles that may impact viral RLU titres.
16. Optional step (Day 4): 2 ml fresh culture media may be added to cells to allow a second harvest 18-24 h later (Day 5) by adding further DMEM/10% FBS/1% P/S. In this case, extreme care must be taken in initial PV collection (step 14 above) to avoid damage to the cell monolayer by aspirating with a sterile syringe to one side of the well. We have observed that cells in poor health after the first harvest result in significantly lower PV production RLU titres upon the second harvest.

Note: A control pseudotype virus can be created by following the steps outlined above but omitting the pCAGGS-SARS-CoV spike construct. This produces particles that do not express a viral surface glycoprotein (Delta S PV control).

B. Preparation of target cells for titration and neutralisation assays (1 day)

Note: SARS-CoV-2 virus host cell entry depends on receptor ACE2 and serine protease TMPRSS2 for S protein priming (Hoffmann et al., 2020). HEK293T/17 are transfected with ACE2 and TMPRSS2 plasmids to be used as optimal target cells for SARS-CoV-2 PV entry. It is therefore essential to pre-transfect the cells whether a PV titration or neutralisation assay is planned.

1. HEK 293T/17 cells should be seeded into a 10 cm cell culture dish at a ratio that will deliver 70-90% confluence at the time of transfection. Typically seeding 2 × 10⁶ cells/plate and incubated overnight at 37°C will achieve this level of confluency.
2. DMEM/10% FBS/1% P/S and Opti-MEM® should be pre-warmed to 37°C using a water bath or an incubator.
3. Prepare one sterile 1.5 ml microcentrifuge tube for each cell culture dish which will be used for transfections.
4. Add the following plasmid constructs (in a total volume of 15 µl in molecular biology grade water) for transfection (DNA mix):
pCDNA3.1+-ACE2 cell entry receptor: 2 µg
pCAGGS-TMPRSS2 serine protease: 150 ng
Note: Plasmid amounts required are determined by prior optimization.
5. Add 100 µl Opti-MEM® to the plasmid DNA mix.

6. Incubation step: Mix tube by gently flicking and incubate for 5 min at RT.
 7. After incubation, add 9 µl FuGENE® HD directly into the Opti-MEM®/DNA solution tube just below the surface.
 8. Mix by gently flicking the tube.
 9. Incubation step: Incubate the tube at RT for 20 min whilst gently flicking the tube to mix every 3-4 min.
 10. Whilst the transfection mix is incubating, the culture media on the 293T/17 cells should be removed and 10 ml of fresh DMEM/10% FBS/1% P/S added per dish. It is imperative at this point to add culture media slowly to one side of the well to avoid detaching the adherent cell monolayer.
 11. After 20 min incubation, pipette the DNA/Opti-MEM®/ FuGENE® HD solution onto the 293T/17 cells by adding dropwise throughout the total surface area of the dish. Swirl the 10 cm cell culture dish(es) gently to ensure an even dispersal of reagent mix.
 12. Incubation step: Incubate the plate at 37°C, 5% CO₂ overnight (16-24 h). In our hands, this incubation time results in sufficient overexpression of cell entry receptors (ACE2/TMPRSS2) for SARS-CoV-2 PV entry.
- C. SARS-CoV-2 PV Titration for the calculation of relative light units (RLU)/ml
- Notes:*
- a. *Titration consists of transduction of reporter (in this case firefly luciferase, but GFP may be used) into target cells mediated by the viral glycoprotein expressed on the viral pseudotype (SARS-CoV-2 spike). Target cells are transfected with ACE2 and TMPRSS2 24 h prior to the titration. Efficiency of the transfection of ACE2/TMPRSS2 was consistently reproducible between several operators and different laboratories; in the rare occasion of a suboptimal transfection, the PV titers dropped dramatically, making results unusable.*
 - b. *Controls for titration are provided via the inclusion of 'cell only' and 'Delta S' (no Spike plasmid) columns. Positive control for transduction can be provided via a PV bearing the Vesicular stomatitis virus G protein (VSV-G), which utilises a ubiquitous receptor that results in high RLU titres in all cell lines tested.*
1. In a 96-well white plate, add 50 µl of DMEM/10% FBS/1% P/S to the entire columns of 'cell only' controls (see Figure 3, columns 9-11).
 2. Add 50 µl of DMEM/10% FBS/1% P/S from rows B to H that are to contain PV or Delta S control virus.
 3. Add 100 µl of SARS-CoV-2 pseudotype virus supernatant to each well of row A (excluding cell only control columns) and add 100 µl of Delta S to column 12 (see Figure 3).

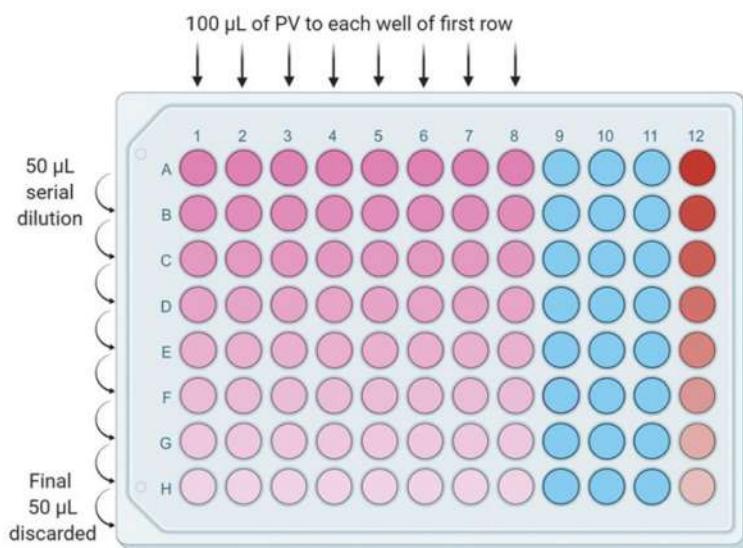


Figure 3. 96-well plate set-up for pseudotype titration. Serial dilution step showing addition of 100 μL of pseudotype virus supernatant to each well of row A and dilution of 50 μL taken from this well to row B. This process is continued to end of plate (row H), at which point the final 50 μL is discarded. Delta S control is indicated in red (column 12), and cell only controls are indicated in blue (columns 9-11). One set of pipette tips can be used per dilution series (plate).

4. With the aid of a 12-channel pipette, remove 50 μL from row A virus-containing wells and perform two-fold serial dilutions down all the wells beneath it.
5. With each dilution step, mix at least five times by pipetting up and down and taking care not to produce air bubbles.
6. After completing serial dilution, the final 50 μL from the final well of each column should be discarded.

Note: At this point, each well should contain 50 μL of PV supernatant only (row A) or mixed and diluted with DMEM (rows B to H).

7. Prepare a plate of susceptible target cells (HEK 293T/17 expressing ACE2 and TMPRSS2 for SARS-CoV-2 PV):
 - a. Remove culture media from plate.
 - b. Wash the plate twice with 2 ml of PBS to one side of the dish to avoid cell detachment and discard.
 - c. Add 2 ml of trypsin to the plate and put the plate into the incubator until the cells are detached (about 5 min).
 - d. After cells have detached, add 6 ml of DMEM/10% FBS/1% P/S to the plate to quench trypsin activity and resuspend cells gently.
 - e. Count cells using TC20™ Automated Cell Counter or counting-chamber slide and add 1 \times 10⁴ cells in a total volume of 50 μL to each well.
8. Centrifuge plate for 1 min at 500 rpm if there are visible droplets on the sides of the wells.
9. Incubate the plate for 48 h at 37°C 5% CO₂.

10. Read plate using Bright Glo™ luciferase assay system on a GloMax® Navigator Microplate Luminometer (or equivalent) by removing the medium from all wells and adding 25 µl of a 1:1 mix of PBS:Bright Glo™ luciferase assay reagent.

11. Data analysis

RLU readings from the luminometer are multiplied to get RLU/ml by the dilution factor of each well (20×, 40×, 80×, 160×, 320×, 640×, 1,280×, 2,560×). The mean of all 8 RLU/ml values is used as the final value reported for that column in the titration step. A linear relationship should be observed between RLU values and PV dilution, with values decreasing by 50% after each 1:2 dilution. Care should be taken to check this linear relationship before multiplication, as this inherently can lead to false production titres being calculated (Table 1).

Table 1. Analysis of SARS-CoV-2 PV titration data and calculation of RLU/ml. RLU values (Top panel) are multiplied to give an RLU/ml value for each of the dilution points (Bottom panel). The mean/average is then calculated from all eight dilution points (expressed as RLU/ml). Care must be taken to observe a linear relationship between dilution factor (X factor) and RLU, or multiplication can lead to inflated RLU/ml values. Analysis is performed on Microsoft Excel (Microsoft®). For SARS-CoV-2 PV optimal production titre in this experiment (3.1×10^8 RLU/ml) is achieved when both ACE2 and TMPRSS2 are present in the target cells (column 8).

	ACE2			TMPRSS2			TMPRSS2 + ACE2			HEK293T/17		
	+ PV	only	Cell only	+ PV	only	Cell only	+ PV	only	Cell only	+ PV	only	
Dilution Factor	5.93E+04	2.00E+02	1.10E+02	3.33E+03	5.00E+01	5.00E+01	9.70E+02	1.40E+07	1.48E+04	1.10E+02	2.03E+03	3.04E+03
	5.73E+04	5.00E+01	4.00E+01	3.18E+04	2.00E+01	3.00E+01	8.20E+02	6.27E+06	6.50E+02	5.00E+01	6.00E+01	4.00E+01
	1.41E+04	7.00E+01	4.00E+01	5.10E+02	5.00E+01	1.00E+02	5.00E+02	3.45E+06	5.50E+02	4.00E+01	6.00E+01	4.00E+01
	2.40E+04	8.00E+01	2.00E+01	3.00E+01	2.00E+01	3.00E+01	2.50E+02	1.81E+06	2.60E+02	3.00E+01	3.00E+01	3.00E+01
	8.26E+03	5.00E+01	2.00E+01	1.00E+02	4.00E+01	2.00E+01	1.30E+02	1.11E+06	1.40E+02	5.00E+01	3.00E+01	3.00E+01
	3.00E+01	2.00E+01	2.00E+01	6.00E+01	4.00E+02	5.00E+01	6.00E+01	4.23E+05	8.00E+01	5.00E+01	2.00E+01	1.00E+01
	2.00E+01	6.00E+01	4.00E+01	3.00E+01	4.00E+01	4.00E+01	6.00E+01	3.47E+05	7.00E+01	4.00E+01	3.00E+01	3.00E+01
	4.00E+01	3.00E+01	3.00E+01	2.00E+01	4.00E+01	3.00E+01	6.00E+01	1.24E+05	3.00E+01	3.00E+01	5.00E+01	1.00E+01
	ACE2			TMPRSS2			TMPRSS2 + ACE2			HEK293T/17		
	+ PV	only	Cell only	+ PV	only	Cell only	+ PV	only	Cell only	+ PV	only	
	1.19E+06	4.00E+03	1.10E+02	6.66E+04	1.00E+03	5.00E+01	9.70E+02	2.81E+08	2.97E+05	1.10E+02	4.06E+04	6.08E+04
	2.29E+06	2.00E+03	4.00E+01	1.27E+06	8.00E+02	3.00E+01	8.20E+02	2.51E+08	2.60E+04	5.00E+01	2.40E+03	1.60E+03
	1.13E+06	5.60E+03	4.00E+01	4.08E+04	4.00E+03	1.00E+02	5.00E+02	2.76E+08	4.40E+04	4.00E+01	4.80E+03	3.20E+03
	3.83E+06	1.28E+04	2.00E+01	4.80E+03	3.20E+03	3.00E+01	2.50E+02	2.90E+08	4.16E+04	3.00E+01	4.80E+03	4.80E+03
	2.64E+06	1.60E+04	2.00E+01	3.20E+04	1.28E+04	2.00E+01	1.30E+02	3.55E+08	4.48E+04	5.00E+01	9.60E+03	9.60E+03
	1.92E+04	1.28E+04	2.00E+01	3.84E+04	2.56E+05	5.00E+01	6.00E+01	2.71E+08	5.12E+04	5.00E+01	1.28E+04	6.40E+03
	2.56E+04	7.68E+04	4.00E+01	3.84E+04	5.12E+04	4.00E+01	6.00E+01	4.44E+08	8.96E+04	4.00E+01	3.84E+04	3.84E+04
	1.02E+05	7.68E+04	3.00E+01	5.12E+04	1.02E+05	3.00E+01	6.00E+01	3.18E+08	7.68E+04	3.00E+01	1.28E+05	2.56E+04
RLU/mL	1.40E+06	2.59E+04	4.00E+01	1.93E+05	5.39E+04	4.38E+01	3.56E+02	3.10E+08	8.39E+04	5.00E+01	3.02E+04	1.88E+04

12. Results (Figure 4)

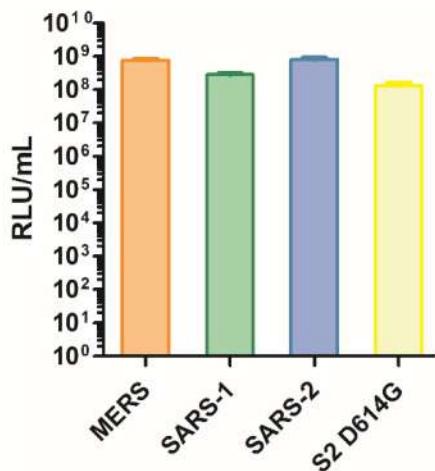


Figure 4. Production of SARS-CoV-2 PV (and D614G variant) and comparison with SARS-CoV (Temperton et al., 2005) and MERS-CoV (Grehan et al., 2015). RLU/ml production titres shown for MERS-CoV, SARS-CoV, SARS-CoV-2, and SARS-CoV-2 (D614G). SARS-CoV, SARS-CoV-2, and SARS-CoV-2 PV titrated on 293T/ACE2/TMPRSS2 cells, MERS-CoV PV titrated on Huh7 cells (Grehan et al., 2015). Alternatively, MERS-CoV-2 PV may be titrated on 293T cells that have been pre-transfected with a DPP4 plasmid.

D. Pseudotype based neutralisation assay (pMN)

Note: pMN is the Inhibition of PV mediated transduction via an antibody (or inhibitor) directed against the SARS-CoV-2 S glycoprotein.

1. In a 96-well white plate with the aid of a multichannel pipette, add 50 µl of DMEM/10% FBS/1% P/S to rows B to H, columns 1-12.
2. Add known amount of antibody (for example, 5 µl convalescent sera or mAb at 10 µg/ml) into wells of row A, columns 2-10 in a total volume of 100 µl DMEM/10% FBS/1% P/S. Add known amount (e.g., 5 µl) of positive and negative antisera into wells A11 and A12 as controls.
3. Remove 50 µl from row A wells and perform two-fold serial dilutions down all the wells beneath it.
4. With each dilution step, use a multichannel pipette to mix 5-10 times by pipetting up and down and taking care not to produce air bubbles.
5. After completing serial dilution, the final 50 µl from the final well of each column should be discarded.

Note: At this point, each well should contain 50 µl of mixed DMEM and serial dilutions of antibody/inhibitor.

6. Centrifuge plate for 1 min at 500 rpm to ensure no inhibitor or liquid is located on the walls of the well.
7. Using data obtained from the titration (Table 1), calculate the amount of DMEM required to dilute

your SARS-CoV-2 PV to obtain 1×10^6 RLU in 50 μ l, with a total volume of 5 ml. For example, with an RLU/ml of 1×10^8 , 1 ml of PV should be mixed with 4 ml of DMEM.

8. Mix this diluted PV solution in a pipette basin using the multichannel pipette, and aliquot 50 μ l into each well on the plate, with the exception of wells A6-A12 (cell only control). A1-A6 will serve as PV only control.
9. Centrifuge plate for 1 min at 500 rpm to ensure no virus is left on the walls of the well. This is critical to avoid virus spikes in the downstream data.
10. Incubate the plates for 1 h at 37°C 5% CO₂, allowing time for the antibody/inhibitor to bind the SARS-CoV-2 glycoprotein.
11. Prepare a plate of susceptible target cells (HEK 293T/17 for SARS-CoV-2 PV), preferentially transfected 24 h before with ACE2 and TMPRSS2:
 - a. Remove culture media from plate.
 - b. Wash the plate twice with 2 ml of PBS to one side of the dish to avoid cell detachment and discard.
 - c. Add 2 ml of trypsin to the plate and put the plate into the incubator until the cells are detached.
 - d. After cells have detached add 6 ml of DMEM/10% FBS/1% P/S to the plate to quench trypsin activity and resuspend cells gently.
 - e. Count cells using TC20™ Automated Cell Counter or counting-chamber slide and add 1×10^4 cells in a total volume of 50 μ l to each well.
12. Incubate the plate for 48-72 h at 37°C 5% CO₂.
13. Read plate using Bright Glo™ luciferase assay system on a GloMax® Navigator Microplate Luminometer (or equivalent) by removing the medium from all wells and adding 25 μ l of a 1:1 mix of PBS:Bright Glo™ luciferase assay reagent.
14. From the raw data provided by the luminometer, calculate the half maximal inhibitory concentration (IC50) neutralising antibody titres using the previously optimised protocol from our group, which is currently being used by the NIBSC and other stakeholders (Ferrara and Temperton, 2018).
15. Results are shown in Figure 5.

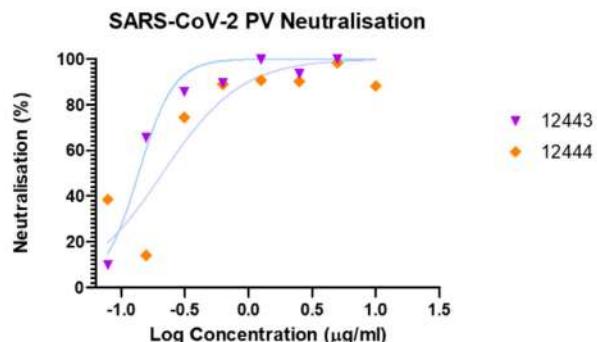
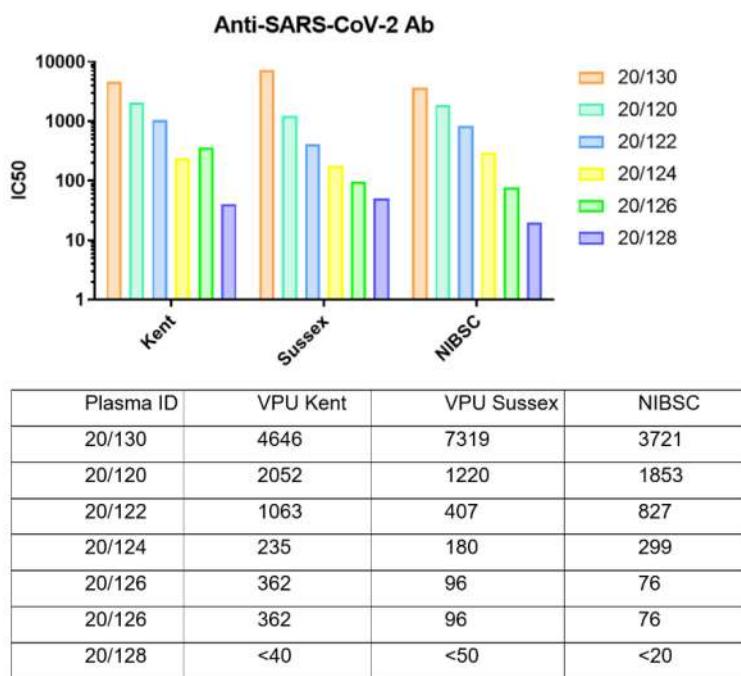


Figure 5. Neutralisation of SARS-CoV-2 PV entry into target cells (HEK 293T/17 expressing ACE2 and TMPRSS2) using reference plasma panel from NIBSC or neutralising mAbs from Native Antigen. Top panel: bar chart showing IC50s for panel of convalescent plasma run in three different laboratories. Middle panel: IC50 data for panel of plasma run in three different laboratories. Bottom panel: mAb neutralisation of SARS-CoV-2 pseudotypes (Native Antigen MAB12443 and MAB12444).

E. Storage of SARS-CoV-2 PV at different temperatures

This protocol allows simulation of different temperature conditions and shipment duration from lab to lab for collaborative studies. UK to UK shipment will routinely be <24 h, UK to EU shipment will be <72 h. For international destination shipments that may be >72 h, dry ice shipment or lyophilisation is recommended.

1. Place eight aliquots of 100 μ l of SARS-CoV-2 PV (prepared above) at different storage temperature for 24 h or 72 h prior to titration (two aliquots each were kept at RT, +4°C, -20°C or -80°C) (see Figure 6 for results).
2. After 24 h, add four aliquots (one aliquot for each different storage temperature) to row A wells

of a 96-well white plate to perform a titration. See Procedure C on how to perform a titration.

3. After 72 h, add the remaining four aliquots at different temperatures to row A wells of a 96-well white plate to perform a titration. See Procedure C on how to perform a titration.

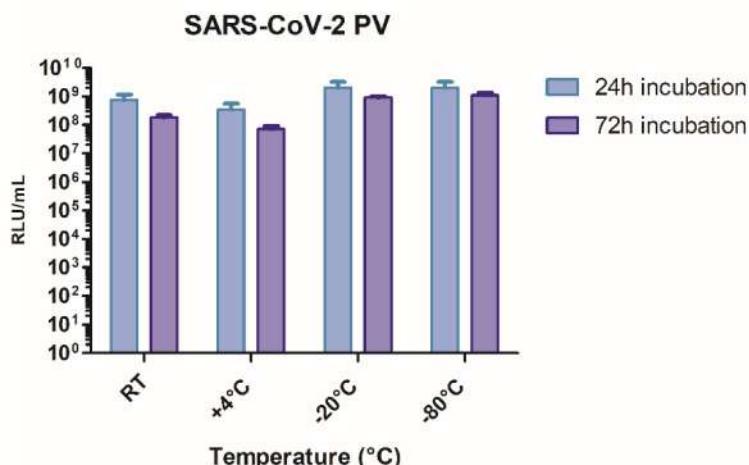


Figure 6. Short term storage of SARS-CoV-2 PV at different laboratory temperatures. PV were stored at RT, +4°C, -20°C and -80°C for 24 or 72 h and then titrated. The PV aliquots kept at -80°C act as the positive control for titration experiment. -80°C is routinely used for long term storage of PV.

F. Lyophilisation of SARS-CoV-2 PV

We have previously shown that lentiviral pseudotypes with influenza, filovirus, or lyssavirus glycoproteins on their surface can be lyophilised for long term storage and shipping (Mather et al., 2014). This methodology is applicable equally to coronavirus pseudotypes.

1. Dissolve sucrose in DPBS to make a 1 M cryoprotectant solution. Syringe sterilise through a 0.45 µm filter.
2. Produce a stock of detached, standard microtube lids with single hole piercings using a sterile syringe needle (allowing vapour to escape during sample freeze-drying).
3. Add 100 µl of high-titre SARS-CoV-2 PV produced above to 100 µl of cryoprotectant in a low-retention microtube. Vortex mix for 5 s.
4. Leave low-retention tube lid open, replacing with a pierced lid.
5. Place samples in plastic racks in a -80°C freezer for a minimum of 1 h.
6. Switch on power to freeze dryer, then vacuum pump.
7. Press MAN button on freeze dryer
8. When temperature reaches -50°C, place sample racks in upper chamber and close valve.
9. Press VACUUM button and wait until series of orange lights have lit up until final green light is illuminated (typically 0.035 mBar/3.5 Pa). Leave overnight (16-20 h).

Note: Samples should not bubble or move up microtubes during lyophilisation (see Figure 7).

10. Open top valve slowly to equalise chamber to atmospheric pressure.

11. Switch off VACUUM, then MAN buttons and finally power to vacuum pump and freeze dryer.
12. Retrieve samples, remove pierced lids, and close microtubes with original lids
13. Store in freezer (-80°C or -20°C). For stability testing in other conditions, monitoring can be conducted using a temperature/humidity meter.



Figure 7. Lyophilised SARS-CoV-2 PV pellets within the sample drying chamber

G. Titration of reconstituted SARS-CoV-2 PV immediately after lyophilisation and employment in a neutralisation assay

1. SARS-CoV-2 PVs were lyophilised as described above (Procedure F).
2. Immediate reconstitution of the lyophilised pellets was done by adding 100 µl of either DPBS or complete cell culture media to compare the influence of the reconstitution solution in a titration.
3. Recommended step: mix well with the help of a vortex mixer and incubate for 10 min at RT before further application to ensure entire resuspension of the pellets.
4. Perform a titration as described above (Procedure C) using the reconstituted aliquots.
5. Perform a pMN as described above (Procedure D) using lyophilised and reconstituted SARS-CoV-2 pellets (in this case, DMEM was chosen as reconstitution solution).
6. Results (Table 2, Figure 8, Figure 9)

Table 2. Titre comparison of lyophilised SARS-CoV-2 PV reconstituted either in DPBS or in complete cell culture media (DMEM). Viral titres were compared to their non-lyophilised counterpart (CTRL). Analysis of SARS-CoV-2 PV titration data and calculation of RLU/ml has been carried out as in Table 1.

Note: Consider the cytotoxic effect of the cryoprotectant (sucrose). Therefore, the first 4 dilution points of the lyophilised samples are not taken into account to calculate the mean/average (final RLU/ml).

Dilution Factor	LYOPHILISED						LYOPHILISED						CTRL		
	S2 DPBS	S2 DPBS	Cell only	Cell only	S2 DMEM	S2 DMEM	Cell only	Cell only	Cell only	Cell only	S2	S2			
	20	1.00E+02	1.30E+02	7.00E+01	9.00E+01	9.00E+01	5.00E+01	9.00E+01	4.00E+01	9.00E+01	5.42E+03	2.39E+07	2.17E+07		
40	1.40E+02	1.40E+02	7.00E+01	4.00E+01	1.10E+02	2.60E+02	3.30E+02	1.20E+02	7.00E+01	5.56E+03	1.48E+07	1.79E+07			
80	1.93E+05	3.82E+05	3.00E+02	6.00E+01	3.10E+02	5.95E+05	4.33E+05	3.00E+02	6.00E+01	3.55E+03	8.31E+06	9.22E+06			
160	1.52E+06	1.86E+06	6.30E+02	4.00E+01	8.30E+02	2.74E+06	1.74E+06	6.80E+02	7.00E+01	1.97E+03	5.81E+06	5.94E+06			
320	2.05E+06	1.73E+06	7.30E+02	7.00E+01	9.00E+02	2.14E+06	1.54E+06	6.00E+02	4.00E+01	1.07E+03	2.23E+06	3.02E+06			
640	9.17E+05	1.23E+06	5.40E+02	3.00E+01	4.90E+02	1.05E+06	1.26E+06	4.90E+02	6.00E+01	4.50E+02	1.40E+06	1.47E+06			
1280	5.35E+05	4.88E+05	2.30E+02	3.00E+01	2.70E+02	6.28E+05	4.65E+05	2.90E+02	5.00E+01	3.40E+02	6.91E+05	7.57E+05			
2560	3.07E+05	1.97E+05	9.00E+01	4.00E+01	1.30E+02	3.66E+05	3.53E+05	1.10E+02	4.00E+01	1.20E+02	5.25E+05	5.02E+05			
LYOPHILISED															
Dilution Factor	S2 DPBS	S2 DPBS	Cell only	Cell only	S2 DMEM	S2 DMEM	Cell only	Cell only	Cell only	S2	S2	CTRL			
	2.00E+03	2.60E+03	7.00E+01	9.00E+01	9.00E+01	1.00E+03	1.80E+03	4.00E+01	9.00E+01	5.42E+03	4.78E+08	4.33E+08			
5.60E+03	5.60E+03	7.00E+01	4.00E+01	1.10E+02	1.04E+04	1.32E+04	1.20E+02	7.00E+01	5.56E+03	5.92E+08	7.17E+08				
1.54E+07	3.05E+07	3.00E+02	6.00E+01	3.10E+02	4.76E+07	3.46E+07	3.00E+02	6.00E+01	3.55E+03	6.65E+08	7.38E+08				
2.42E+08	2.97E+08	6.30E+02	4.00E+01	8.30E+02	4.39E+08	2.78E+08	6.80E+02	7.00E+01	1.97E+03	9.30E+08	9.50E+08				
6.57E+08	5.54E+08	7.30E+02	7.00E+01	9.00E+02	6.85E+08	4.92E+08	6.00E+02	4.00E+01	1.07E+03	7.12E+08	9.66E+08				
5.87E+08	7.90E+08	5.40E+02	3.00E+01	4.90E+02	6.71E+08	8.03E+08	4.90E+02	6.00E+01	4.50E+02	8.96E+08	9.43E+08				
6.84E+08	6.25E+08	2.30E+02	3.00E+01	2.70E+02	8.04E+08	5.95E+08	2.90E+02	5.00E+01	3.40E+02	8.84E+08	9.69E+08				
7.87E+08	5.04E+08	9.00E+01	4.00E+01	1.30E+02	9.36E+08	9.04E+08	1.10E+02	4.00E+01	1.20E+02	1.34E+09	1.29E+09				
RLU/mL	6.79E+08	6.18E+08	3.33E+02	5.00E+01	3.91E+02	7.74E+08	6.99E+08	3.29E+02	6.00E+01	2.31E+03	8.13E+08	8.75E+08			

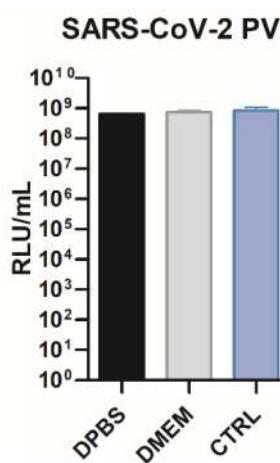


Figure 8. RLU/ml production titres of lyophilised SARS-CoV-2 PV reconstituted either in DPBS or DMEM, compared with their non-lyophilised counterpart (CTRL).

Plasma ID	VPU Kent
20/130	2214
20/124	107
20/126	79
20/128	<40

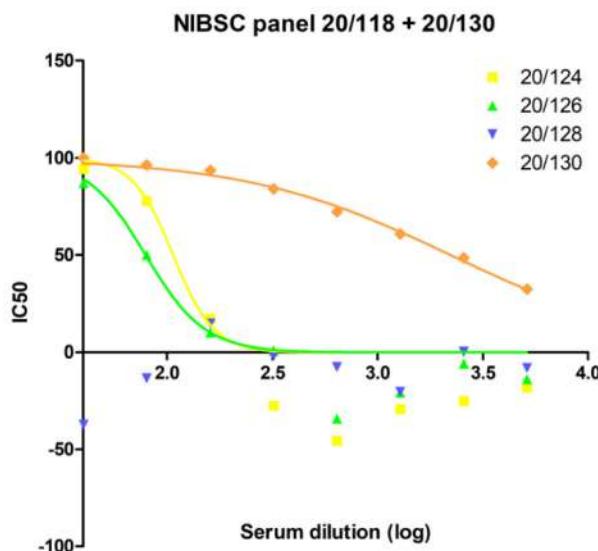


Figure 9. Neutralisation of SARS-CoV-2 viral pseudotype using reference plasma panel from NIBSC. Top panel: IC50 data for panel of plasma. Bottom panel: plasma neutralisation of SARS-CoV-2 PV. These results accomplished by using lyophilised and reconstituted SARS-CoV-2 PV are comparable with Figure 5 (VPU Kent), where non-lyophilised PV was employed. Note: the reconstituted pellets containing cryoprotectant are diluted before adding the PV to the pseudotype virus neutralisation assay (PVNA) (as normal procedure). Therefore, cytotoxicity was not encountered. Neutralisation was tested on four out of six of the original NIBSC plasma panel.

H. Replacement of SARS-CoV-S wild-type cassette in pCAGGS or subcloning of SARS-CoV-2-S wild-type cassette into alternative backbone plasmids (application for new Spike variants or coronaviruses)

Separately restriction digest donor (2 µg) and recipient plasmids (1 µg) using appropriate enzymes according to manufacturer's instructions (cut either side of S gene, but not internally). For the former, use Anza kit Red Buffer (with gel loading dye); for the latter, use Standard Buffer and nuclease free water for both.

1. Pour a 50 ml 1% TAE Ultrapure agarose gel, adding 50 µl SYBR Safe when microwaved gel mix cools to ~50°C.
2. Load donor plasmid digest (and DNA ladder) onto gel and run for 1 h at 80 V.

3. Place gel on transilluminator (70% intensity setting) to visualise DNA and carefully excise Spike gene gel fragment using a clean scalpel.
4. Purify gene with the QIAquick Gel Extraction kit according to manufacturer's instructions. Elute in nuclease free water.
5. Purify recipient plasmid with the QIAquick PCR Purification kit according to manufacturer's instructions. Elute in nuclease free water.
6. Measure concentration of purified recipient plasmid and Spike gene fragment via Nanodrop
7. Perform Anza ligation reaction according to manufacturer's instructions using 50 ng of recipient plasmid and a 1:3 molar ratio of S gene DNA.
8. Transform ligation into Subcloning Efficiency *E. coli* cells according to manufacturer's instructions, using LB broth as a culture medium.
9. Prepare LB agar plates containing appropriate antibiotic (*i.e.*, ampicillin, Sigma-Aldrich 1,000× stock 100 mg/ml solution catalog number A5354).
10. Plate out and spread each 100 µl transformed cell culture on ampicillin agar plates. Incubate at 37°C overnight.
11. Pick discrete colonies (Figure 10) onto a grid on a new ampicillin plate. Incubate 37°C overnight.

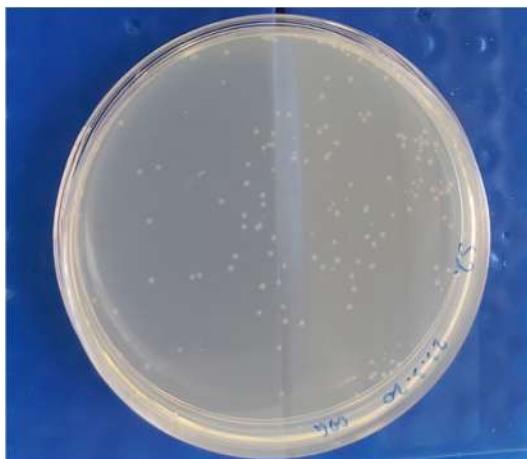


Figure 10. Picture of discrete bacterial colonies

12. Pick cells from each colony into a separate 0.2 ml microtube in 50 µl nuclease free water. Lyse cells in thermocycler at 94°C for 3 min.
13. Screen plasmid DNA in each colony lysate for presence of S gene insert using appropriate PCR primers targeting arms of particular recipient vector. Use 5 µl lysate, 12.5 µl PCR Master Mix, 7.5 µl nuclease free water. Typical thermocycler program: 94°C for 3 min, then 25 cycles of [94°C for 1 min, 50°C for 1 min, 72°C for 2 min], 72°C for 5 min.
14. Run on agarose gel as above. Photograph gel with gel imaging system and identify positive clones with S gene insert (~3.75 kbp).
15. Inoculate 5 ml LB-Amp broth cultures with positive clone cells from grid. Incubate in 37°C shaking incubator overnight.

16. Purify S gene plasmid clone DNA using a miniprep kit.
17. Sequence verify using commercial service (Eurofins, for example).
18. Once verified, the new SARS-CoV-S plasmid can be incorporated into the PV production protocol above (Procedure A).

Conclusions

1. The protocol outlined provides a rapid and consistent method for the generation of high-titre viral pseudotype particles expressing the SARS-CoV-2 spike protein suitable for further downstream R&D applications. Production titres obtained are equivalent to those obtained for SARS-CoV and MERS-CoV (Figure 4).
2. Efficient knock-down (neutralisation) of SARS-CoV-2 PV entry into target cells using human convalescent plasma and mAbs demonstrates potential utility for vaccine immunogenicity and mAb/antiviral screening. The use of readily available reagents should facilitate increased reproducibility, both intra- and inter-laboratory, as demonstrated in Figure 6. These pseudotypes can be stored at a range of laboratory temperatures (Figure 6) and may be lyophilised for long term storage and easy global distribution (Figure 7). The plug and play nature of the pseudotype system makes it straightforward to swap out the Spike cassette for a new variant Spike of SARS-CoV-2 or indeed for another coronavirus. It is hoped that this suite of protocols will facilitate the wide-scale adoption of pseudotype technologies for vaccine and therapeutic R&D on emerging coronaviruses of human and animal concern.

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Competing interests

The authors declare no conflicts of interest.

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A Nucleocapsid-based Transcomplementation Cell Culture System of SARS-CoV-2 to

Recapitulate the Complete Viral Life Cycle

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[Abstract] The ongoing COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As this virus is classified as a biosafety level-3 (BSL-3) agent, the development of countermeasures and basic research methods is logistically difficult. Recently, using reverse genetics, we developed a BSL-2 cell culture system for production of transcription- and replication-component virus-like-particles (trVLPs) by genetic transcomplementation. The system consists of two parts: SARS-CoV-2 GFP/ΔN genomic RNA, in which the nucleocapsid (N) gene, a critical gene for virion packaging, is replaced by a GFP reporter gene; and a packaging cell line for ectopic expression of N (Caco-2-N). The complete viral life cycle can be recapitulated and confined to Caco-2-N cells, with GFP positivity serving as a surrogate readout for viral infection. In addition, we utilized an intein-mediated protein splicing technique to split the N gene into two independent vectors and generated the Caco-2-N^{intein} cells as a packaging cell line to further enhance the security of this cell culture model. Altogether, this system provides for a safe and convenient method to produce trVLPs in BSL-2 laboratories. These trVLPs can be modified to incorporate desired mutations, permitting high-throughput screening of antiviral compounds and evaluation of neutralizing antibodies. This protocol describes the details of the trVLP cell culture model to make SARS-CoV-2 research more readily accessible.

Keywords: SARS-CoV-2, trVLP, Reverse genetics, BSL-2, Nucleocapsid, Transcomplementation

[Background] The COVID-19 pandemic caused by SARS-CoV-2 still rages around the world, threatening public health and the global economy (Wang et al., 2020). Several reverse genetic systems have been reported for generating SARS-CoV-2 replicons and recombinant virus for basic virology research and antiviral development (Hou et al., 2020; Xie et al., 2020; Zhang et al., 2021a and 2021b). However, as SARS-CoV-2 is classified as BSL-3 pathogen, experiments involving authentic virus are restricted to BSL-3 laboratories, which hinders basic research and antiviral discovery. Pseudotyped virus and replicon systems of SARS-CoV-2 that can be used in BSL-2 laboratories have been developed but are limited to studying viral entry and replication, respectively (Nie et al., 2020; Zhang et al., 2021b). Therefore, a cell culture model for SARS-CoV-2 that could safely recapitulate the entire viral life cycle in a BSL-2 facility is urgently needed (Rome and Avorn, 2020).

Recently, we developed such a system in which transcription- and replication-component SARS-CoV-2 virus-like particles (SARS-CoV-2 trVLPs) can be generated (Ju et al., 2021). We replaced the N gene

in the SARS-CoV-2 genome with a GFP reporter gene and then provided N *in trans* in Caco-2 cells ectopically expressing this gene (Caco-2-N). Transducing the Caco-2-N cells with the SARS-CoV-2 GFP/ΔN genome thus allows for the production of trVLPs, which can complete the entire life cycle exclusively in the Caco-2-N packaging cells. In normal cells, these trVLPs can only complete a single-round infection as the packaged viral genome lacks the N gene, which is critical for viral particle assembly. Thus, this system can be safely utilized in BSL-2 level laboratories for SARS-CoV-2 research. In addition, the GFP reporter provides a convenient surrogate readout for virus infection, which facilitates the use of this system for neutralizing antibody determination and high-throughput antiviral screening.

Genetic manipulation of this SARS-CoV-2 trVLPs system is not trivial due to the large size of the viral genome (~30 kb) and presence of multiple toxic elements (Almazan *et al.*, 2014; Xie *et al.*, 2021). Herein, we describe technical details of our cell culture system for production of trVLPs. Overall, the engineering process includes three parts: packaging cell line construction, viral genome-length RNA preparation, and trVLP recovery. Using lentiviral transduction, SARS-CoV-2 N protein can be stably expressed in Caco-2 cells to generate the packaging cell line necessary for trVLP production. Besides, an intein-mediated protein trans-splicing approach (Stevens *et al.*, 2017) was utilized to minimize the chance of N gene recombination into the genome of trVLP. The genome-length viral RNA is prepared by *in vitro* transcription of a full-length cDNA template generated by *in vitro* ligation. Briefly, four fragments (A-B, C, D, and E) were designed to cover the entire genome of SARS-CoV-2 GFP/ΔN. Each fragment can be chemically synthesized and then amplified using PCR (**Figure 1**). After PCR amplification, the fragments, which are flanked by a type IIS restriction enzyme recognition site (Bsal), are digested and then ligated *in vitro* to assemble the full-length cDNA of the viral genome. As type IIS restriction enzymes recognize asymmetric DNA sequences and cleave at a defined distance outside of their recognition sequence, the fragments are unidirectionally assembled into the full-length cDNA. A T7 promoter and a poly(A) tail were engineered upstream of fragment A and downstream of fragment E, respectively, ultimately allowing for *in vitro* transcription to produce capped, polyadenylated viral RNA. This genome-length RNA can then be electroporated into the Caco-2-N packaging cell line and trVLPs subsequently collected from the supernatant. Then the trVLPs are amplified and titrated using a tissue-culture infectious dose 50% (TCID₅₀) endpoint dilution assay following the Reed & Muench method (Lindenbach, 2009), and GFP expression is the proxy of trVLP infection. SARS-CoV-2 trVLP can be used for evaluating antivirals and neutralizing antibodies.

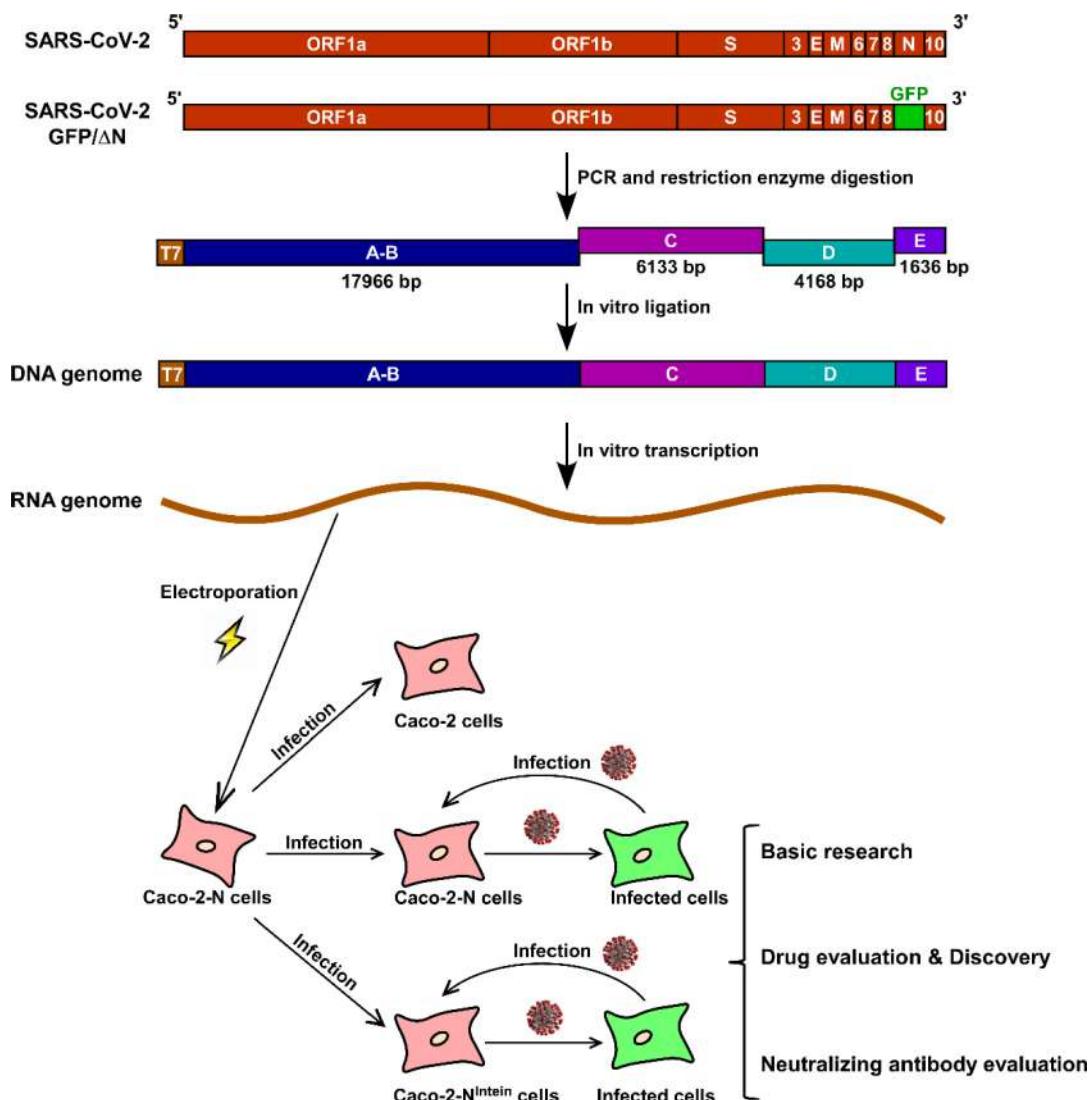


Figure 1. Overview of production of SARS-CoV-2 GFP/ΔN trVLPs. The N gene of SARS-CoV-2 was replaced with the GFP gene, and the cDNA genome divided into four fragments designated as A-B, C, D, and E. Each of these fragments was chemically synthesized and then PCR amplified and assembled by restriction enzyme digestion and *in vitro* ligation to create the full-length cDNA. The full-length RNA genome was generated by *in vitro* transcription of the full-length cDNA. This RNA genome can then be electroporated into the packaging cell line, Caco-2-N, to produce trVLPs. At 24 h post electroporation, the supernatant of electroporated cells is collected and can be used to inoculate Caco-2, Caco-2-N, or Caco-2-N^{Intein} cells. trVLPs can infect and replicate in Caco-2-N or Caco-2-N^{Intein} cells and can be secreted into the supernatant. However, trVLPs only complete a single-round infection in Caco-2 cells due to the absence of viral N protein.

Materials and Reagents

A. Materials

1. Electroporation cuvettes, 4-mm gap (Bio-Rad, catalog number: 1652088)
2. 24-well plate (Thermo Fisher Scientific, catalog number: 142475)
3. 96-well plate (Thermo Fisher Scientific, catalog number: 167008)
4. 0.2-ml PCR tubes (Thermo Fisher Scientific, catalog number: N8010580)
5. 1.5-ml tube (Corning, catalog number: MCT-150-C)
6. 15-ml tube (Thermo Fisher Scientific, catalog number: 339650)
7. 10-cm dish (Thermo Fisher Scientific, catalog number: 150466)

B. Cells

1. Caco-2 cells (ATCC, catalog number: HTB-37)
2. 293T cells (ATCC, catalog number: CRL-3216)
3. Stbl3 competent cells (AlpaLife, catalog number: KTS110L)
4. EPI300 competent cells (Lucigen, catalog number: C300C105)

C. Reagents

1. Dulbecco's Modified Eagle Medium (DMEM; Gibco, catalog number: C11965500BT)
2. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, catalog number: 25200072)
3. Fetal bovine serum (BIOVISTECH, catalog number: SE100-011)
4. Penicillin/streptomycin, 10,000 U/ml (Thermo Fisher Scientific, catalog number: 15140122)
5. DNA transfection reagent Vigofect (Vigorous, catalog number: T001)
6. pMD2G (Addgene, catalog number: 12259)
7. psPAX2 (Addgene, catalog number: 12260)
8. Opti-MEMTM (Gibco, catalog number: 31985070)
9. 5 kb DNA marker (Takara, catalog number: 3428A)
10. 15 kb DNA marker (Takara, catalog number: 3582A)
11. PrimeSTAR[®] Max DNA Polymerase (Takara, catalog number: R045)
12. PrimeSTAR[®] GXL DNA Polymerase (Takara, catalog number: R050)
13. Restriction enzyme Bsal (NEB, catalog number: R0535L)
14. T4 DNA Ligase (NEB, catalog number: M0202L)
15. mMESSAGE mMACHINE[™] T7 transcription kit (Thermo Fisher Scientific, catalog number: AM1344)
16. E.Z.N.A[®] Plasmid DNA Mini Kit I (Omega, catalog number: D6943-02)
17. E.Z.N.A[®] Gel Extraction Kit (Omega, catalog number: D2500-02)
18. Phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.8; Solarbio, catalog number: P1012)
19. ReverTra Ace[®] qPCR RT Kit (Toyobo, FSQ-101)
20. TRIzol[™] Reagent (Thermo Fisher Scientific, catalog number: 15596018)

21. CopyControl™ Induction Solution (Lucigen, catalog number: CCIS125)
22. Polybrene (Sigma, catalog number: TR-1003-G)
23. Dulbecco's Phosphate-Buffered Saline (PBS; Corning, catalog number: 21-031-CVR)

Equipment

1. Water bath (zhybioresources, catalog number: SYG-1210)
2. Centrifuge (Eppendorf, catalog number: 5406000291)
3. Blue Light Gel Imager (Sangon Biotech, catalog number: G500312)
4. NanoDrop™ One/OneC (Thermo Fisher Scientific, catalog number: 701-058108)
5. Gene Pulser Xcell Total Electroporation System (Bio-Rad, catalog number: 1652660)
6. Incubator shaker (Changzhou Huayi, catalog number: THZ-D)

Procedure

Timeline:

- A. Propagation of plasmids bearing SARS-CoV-2 cDNA fragments
Steps 1-2, Chemical transformation (2 h) and colony screen (overnight).
Step 3, Plasmid preparation: 1 h.
- B. Construction of Caco-2-N and Caco-2-N^{intein} cell line
Steps 1-2, Lentivirus packaging (3) and transduction (3 d).
- C. Preparation of DNA fragment by PCR
Steps 1-3, PCR amplify fragments A-B (6 h), C, D and E (2-3 h).
Steps 4, Purification of PCR products by phenol-chloroform extraction: 1-2 h.
- D. Generation of genome-length cDNA by restriction enzyme digestion and *in vitro* ligation
Steps 1-2, Fragments C, D, and E digestion (5 h) and purification (1-2 h).
Step 3, Fragments C, D, and E *in vitro* ligation: 24 h.
Step 4, PCR amplification of C-D-E (6 h) and purification (1-2 h).
Step 5, Fragments A-B and C-D-E digestion: 5 h.
Step 6, Full-length cDNA assembly by *in vitro* ligation: 48 h.
- E. Generation of viral genome-length RNA by *in vitro* transcription (IVT)
Step 1, PCR amplification of N gene (1 h) and purification (1-2 h).
Steps 2-8, *In vitro* transcription: 1 d.
- F. Electroporation and trVLP recovery
Steps 1-4, Electroporation: 2 h.
Steps 5-10, trVLP recovery: 3-4 d.
- G. RT-PCR for verification of GFP gene of the trVLP
Step 1, trVLP infection (24 h) and viral RNA extraction: 1-2 h.
Step 2, RT-PCR for amplification of GFP gene (1-2 h) and analysis by agarose electrophoresis (30

min).

H. trVLP titration by TCID₅₀

Steps 1-5, trVLP infection (24 h) and TCID₅₀ calculation (1-2 h).

A. Propagation of plasmids carrying SARS-CoV-2 cDNA fragments

1. Plasmids

The genome of SARS-CoV-2 GFP/ΔN (derived from the Wuhan-Hu-1 strain, GenBank: MN908947) was divided into four fragments designated as A-B, C, D, and E, each obtained by PCR using a chemically synthesized SARS-CoV-2 genome as the template. The fragments were cloned into the pCCI, pMV, or pLVX vectors, resulting in four plasmids: pCCI-A-B, pMV-C, pLVX-D, and pLVX-E. The SARS-CoV-2 N gene with a Flag tag in the N terminal was cloned into the lentivirus vector pLVX-IRES-mCherry, resulting in pLVX-N-Flag-IRES-mCherry that was then used to generate lentivirus. Caco-2 cells transduced with this lentivirus (Caco-2-N) serve as the packaging cell line for producing SARS-CoV-2 trVLPs, as the Caco-2-N cells complement the SARS-CoV-2 GFP/ΔN genome by providing N *in trans*. Meanwhile, N gene was also mutated at 212 amino acid (G212C) and split into two parts. These two parts are fused with intein elements Npu-N and Npu-C (Zettler et al., 2009) separately defining as N^N-Int^N and Int^C-N^C and further cloned into lentiviral vectors, resulting pLVX- N^N-Int^N-IRES-mCherry and pLVX-Int^C-N^C-IRES-puromycin, respectively. These two plasmids encoding either N- or C-terminal of N protein were transduced together to ligate the full-length N in Caco-2 cell for generating another packaging cell line Caco-2-N^{intein}.

2. Transformation

- For each plasmid, thaw EPI 300 or Stbl3 competent cells on ice (see "Note" below) and add 2 µl (approximately 10 ng) of the respective plasmid to the competent cells. Mix by tapping the tube and incubate on ice for 30 min.

Note: For pMV- and pLVX-derived plasmids – including pMV-C, pLVX-D, pLVX-E, pLVX-N-Flag-IRES-mCherry, pLVX-N^N-Int^N-IRES-mCherry, and pLVX-Int^C-N^C-IRES-puromycin – use Stbl3 competent cells for transformation. For pCCI-derived plasmid pCCI-A-B, EPI300 competent cells for transformation.

- Heat shocks the tube containing the cells and plasmid mixture in a 42°C water bath for 90 s, then immediately place back on ice for a 2 min incubation.
- Add 1 ml LB medium to the tube and shake at 37°C for 45 min at 220 rpm using an incubator shaker to recover the cells.
- After recovery, plate 100 µl of the cell culture onto an LB plate supplemented with the appropriate antibiotics (see "Notes" below) and incubate overnight at 37°C.

Notes:

- For selection of Stbl3 bacteria transformed with pMV- and pLVX-derived plasmids, use LB agar plates supplemented with 50 µg/ml ampicillin.*
- For selection of EPI300 bacteria transformed with pCCI-A-B, use LB agar plates*

supplemented with 12.5 µg/ml chloramphenicol.

3. Preparation of plasmids carrying SARS-CoV-2 genes
 - a. After overnight incubation, for each plate, pick 2-3 bacterial colonies and inoculate 4 ml LB supplemented with the appropriate antibiotics (the antibiotic concentration remains the same as on the plates). Incubate overnight (12-16 h) at 37°C with shaking at 220 rpm.
 - b. For pMV- and pLVX-derived plasmids, the overnight culture can directly be used for plasmid preparation. For pCCI-A-B, the bacterial culture should be further induced as follows:
Add 1.5 ml of the overnight culture to a 50 ml flask or tube containing 13.5 ml LB supplemented with 12.5 µg/ml chloramphenicol and 15 µl of CopyControl™ Induction Solution (Lucigen, catalog number: CCIS125), and shake at 220 rpm for 5 h at 37°C.
 - c. Centrifuge the cell cultures by spinning at 4,000 × g for 5 min.
 - d. Discard the supernatant and remove as much residual liquid as possible.
 - e. Following the manufacturer's instructions, use the cell for plasmid extraction via the Plasmid Mini Kit I (Omega).
 - f. At the final step of plasmid extraction, add 50 µl deionized water to the mini-prep column and incubate at RT for 3 min, then centrifuge at 13,000 × g for 1 min to elute the plasmids.
 - g. Determine the concentration and quality of the plasmid prep using a NanoDrop. The plasmids can be used immediately or stored at -20°C until use.

B. Construction of Caco-2-N and Caco-2-N^{intein} cell lines

1. Lentivirus packaging

Prepare vesicular stomatitis virus G protein (VSV-G) pseudotyped lentivirus following this published protocol (Tiscornia et al., 2006). Briefly, use VigoFect DNA transfection reagent to transiently co-transfect HEK293T cells with the third-generation packaging plasmids pMD2G (catalog no. 12259; Addgene), psPAX2 (catalog no. 12260; Addgene), and the transfer vector pLVX-N-Flag-IRES-mCherry. Change the medium 12 h post transfection and collect supernatants at 36, 60, and 84 h post transfection. Pool all the supernatants, pass through a 0.45-µm filter, aliquot, and store at -80°C until needed.

2. Transduction

- a. Seed 1 × 10⁵ Caco-2 cells in a 24-well plate one day prior to infection to ensure 70-80% confluent next day.
- b. When ready for transduction, remove the cell culture medium, add 1 ml of lentivirus supplemented with 10 µg/ml polybrene into the well, and mix by rocking the plate gently. Incubate at 37°C, 5% CO₂ for 48 h.

Notes:

- i. To generate the Caco-2-N cell line, lentivirus packaging pLVX-N-Flag-IRES-mCherry is used for transduction. To generate the Caco-2-N^{intein} cell line, lentivirus packaging pLVX-N^N-Int^N-IRES-mCherry and pLVX-Int^C-N^C-IRES-puromycin are mixed (1:1) and used for transduction of the Caco-2 cells.

ii. The addition of polybrene increases transduction efficiency.

- c. As a measure of transduction efficiency, monitor the mCherry signal daily using a fluorescent microscope. Generally, the mCherry signal can be readily detected at 48 h post infection.
- d. At 48 h post transduction, check the mCherry signal and passage the transduced cells into a 6-well plate and incubate at 37°C, 5% CO₂.
- e. At another 24 h, check the mCherry signal and cell density to make sure at least 90% of cells are mCherry positive, harvesting cells that could be used as the packing cell line for production of trVLPs.

Note: Since these cells are not under selection, the N gene in these Caco-2-derived cell lines can be lost after several passages, which will decrease the packaging efficiency of trVLPs. Thus, re-transduce the Caco-2-N cell lines as needed to maintain the N gene expression level. Alternatively, use a cell sorter to harvest the first 30% of the mCherry-positive cells from the constructed Caco-2-N cell lines if the N expression level is not sufficient (the mCherry positive cells lower than 90%) for trVLP packaging.

C. Generation of SARS-CoV-2 trVLP cDNA by PCR

1. The template, primers, and DNA polymerase in the thermal cycling program for PCR are listed in **Table 1**. The sequences of primers used in this protocol are listed in **Table 2**.

Table 1. Components for PCR reactions to generate select DNA fragments

	A-B	C	D	E
Template	pCCI-A-B	pMV-C	pLVX-D	pLVX-E
Primer pairs	THU2170/THU2177	THU2178/THU2255	THU3824/THU3825	THU2258/THU2261
DNA polymerase	PrimeSTAR® GXL	PrimeSTAR® Max	PrimeSTAR® Max	PrimeSTAR® Max

Table 2. Primers used in this protocol

Primer name	Sequence (5'-3')
THU2020	AGGGGGCCGGTGGTACTAACGTTGTTCTATGAAGACTTTAG
THU2140	TACTGTAATACGACTCACTATAGGATGTCTGATAATGGACCCCCAAATC
THU2141	TTTTTTTTTTTTTTTTTTTTTTGGGAGCTGCCCGGGATCCTTAGG CCTGAGTTGAGTCAGCAC
THU2170	AGAATACTCAAGCTTATCGGATCCATCCGGTCTCGCGAGATTAAACGACT CACTATAGATTAAAGGTTATACCTTCC
THU2177	CCCCTCAGCCTATCGGGCGGGATCCGGATGGTCTCCATCAGACATTATGCAA AGTATGCCTAC
THU2178	TAGAATACTCAAGCTTATCGGATCCATCCGTGATAGAGACCTTATGACAAGT TGCAA
THU2195	CTCTCCCTAGCATTGTTCACTG
THU2255	GGATGGTCTCTCTAGCAGCAATATCACC
THU2258	ATCCGGTCTCAACTAAAATGGTGAGCAAGGGCGAGG
THU2261	GGATGGTCTCCTTTTTTTTTTTTTTTTTTTTTTTTCATTCTCCTA AGAAGC
THU3824	GATCTATTCCGGTGAATTCTCGAGATCCGGTCTCCGTCTCATGATAGAGA CCTTATGACAAGTTGCAATTACAAG
THU3825	GGGGCGGGATCCGCGGCCGCTCTAGAGGATCGTCCTCTTTTTTTTTTT TTTTTTTTTTTTGTCAATTCTCCTAAGAAGC

2. Amplification of fragments A-B

- Set up a 50 μ l PCR reaction using PrimeSTAR® GXL following the manufacturer's instructions. Prepare a 400 μ l reaction master mix and aliquot it into eight 0.2-ml PCR tubes (50 μ l/tube). The components for the PCR reaction are listed in **Table 3**.

Table 3. PCR reaction for amplification of fragment A-B

Component	Volume (μ l)
5× PrimeSTAR® GXL buffer	10
2.5 mM dNTP	4
Forward primer THU2170 (10 μ M)	1
Reverse primer THU2177 (10 μ M)	1
Template pCCI-A-B	10 ng
PrimeSTAR® GXL	1
Deionized water	Up to 50

- Incubate reactions in a thermal cycler according to the program shown in Table 4.

Table 4. Thermal cycling program for PCR reaction to amplify fragment A-B

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	10 s	
Annealing and extension	68°C	10 min	30
Final extension	68°C	10 min	1
	16°C	hold	-

Note: Usually, 30-40 µg of fragment A-B can be recovered from the 400 µl PCR reaction solution.

- c. Examine the PCR products by gel electrophoresis. Mix 2 µl of PCR product and with 6× DNA loading buffer and load the mixture onto a 1% agar gel (**Figure 2A**).

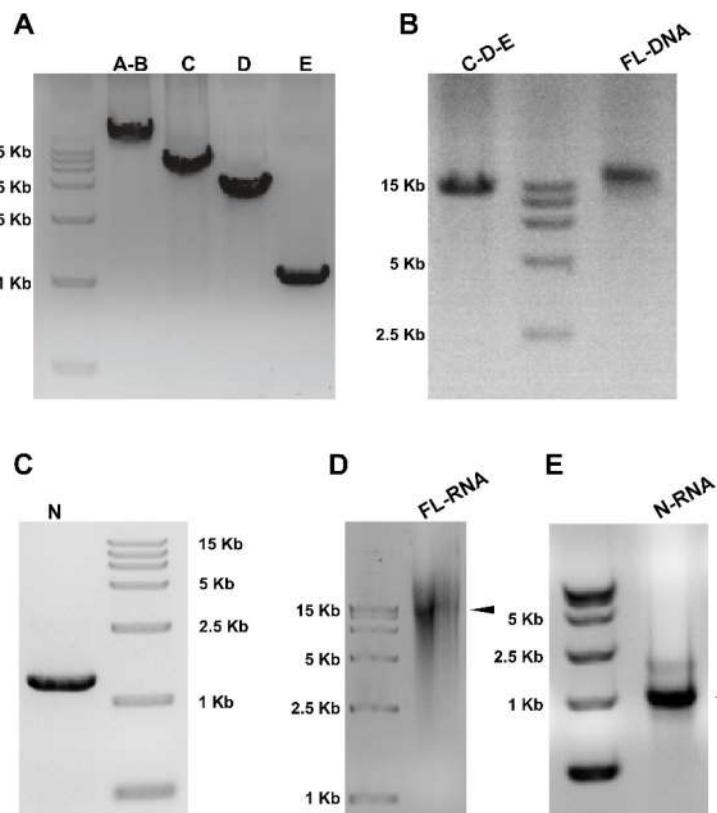


Figure 2. Agarose gel electrophoresis verification of DNA and RNA fragments for trLVPs generation. (A) The genome sequence of trLVPs was divided into four fragments and each fragment PCR amplified. The purified PCR products of each fragment were determined by agarose gel. (B) PCR products of C-D-E and purified ligation products of full-length DNA (FL DNA). (C) Agarose gel analysis of SARS-CoV-2 N gene PCR products. (D) Agarose gel analysis of viral full-length RNA (FL-RNA) and (E) N gene mRNA generated by *in vitro* transcription using the FL DNA genome and N gene PCR products, respectively, as template. The black arrow indicates the FL RNA and N RNA, respectively.

3. Amplification of fragments C, D, and E

- a. Set up a 50 μ l PCR reaction according to the PrimeSTAR[®] Max (Takara) instructions. Prepare a 200 μ l PCR reaction for each fragment and aliquot it into four 0.2 ml PCR tubes, 50 μ l/tube. The components for the PCR reaction are listed in **Table 5**.

Table 5. PCR reaction for amplification of fragments C, D, and E

Component	Volume (μ l)
Forward primer (10 μ M)	1
Reverse primer (10 μ M)	1
Template (pMV-C, pLVX-D, or pLVX-E)	30 ng
2 $×$ PrimeSTAR [®] Max	25
deionized water	Up to 50

- b. Incubate reactions in a thermal cycler according to the program shown in **Table 6**.

Table 6. Thermal cycling program for PCR reaction of fragments C, D, and E

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	15 s	
Annealing	55°C	15 s	30
Extension	72°C	4 min	
Final extension	68°C	5 min	1
-	16°C	hold	-

Note: Usually, approximately 20 μ g of each fragment can be recovered from the 200 μ l PCR reaction solution.

- c. Check the quality of the PCR products by gel electrophoresis. Mix 2 μ l of PCR product and with 6 $×$ DNA loading buffer and load the mixture onto a 1% agar gel (**Figure 2A**).
4. Purification of PCR products by phenol-chloroform extraction
 - a. Pool the PCR products for each fragment into a 1.5-ml tube, add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and mix thoroughly and centrifuge at 13,000 \times g for 1 min at 4°C.
 - b. Transfer the upper aqueous phase into a fresh 1.5-ml tube, add chloroform equal to the aqueous phase volume, mix thoroughly, and centrifuge at 13,000 \times g for 1 min at 4°C.
 - c. Pipet the top layer into a new nuclease-free 1.5-ml tube and add 1/10th volume of 3 M sodium acetate (pH 5.2). Then add isopropanol in a volume ratio of 1:1, mix well and incubate at -20°C for at least 30 min.
 - d. Centrifuge at 13,000 \times g for 15 min at 4°C to pellet the DNA.
 - e. Remove the supernatant, wash the pellet by adding 1 ml 75% ethanol, and centrifuge at

13,000 × g for 5 min at 4°C.

- f. Remove the residual liquid completely, being careful not to remove any pellet.
- g. Air dry the pellet until it is no longer visible and resuspend in 100 µl of nuclease-free water.
- h. Measure the yield and quality of the recovered DNA by using a NanoDrop.
- i. Check the quality of the purified DNA by gel electrophoresis (**Figure 2A**).

D. Generation of genome-length cDNA by restriction enzyme digestion and *in vitro* ligation

Fragments C, D, and E are first digested with Bsa I and ligated by T4 ligase to create the C-D-E ligation product, which is utilized as a template for PCR amplification. Next, the PCR products of fragment A-B and C-D-E are digested with BsAl and ligated to generate the full-length SARS-CoV-2 cDNA.

1. Fragments C, D, and E digestion

- a. Set up an 80-µl digestion reaction for fragments C, D, and E, as shown in **Table 7**.
Fragments C and E are digested with Bsa I.

Table 7. Fragments C, D, and E digestion reactions

Component	Fragment C	Fragment D	Fragment E
10× Cutsmart buffer	5 µl	5 µl	5 µl
DNA fragment	20 µg	20 µg	20 µg
Bsa I	4 µl	4 µl	4 µl
Deionized water	Up to 80 µl	Up to 80 µl	Up to 80 µl

- b. Incubate the fragments C, D, and E digestion reactions at 37°C for 5 h or incubate overnight.
2. Digested DNA fragment purification from agarose gel
 - a. After digestion, add 16 µl 6× DNA loading buffer to each reaction tube, mix thoroughly, and load the mixture onto a 1% agarose gel and run at 180 V for 15 min.
 - b. Visualize the DNA fragment using a Blue Light Gel Imager. Avoid using UV light for visualization as it can cause DNA damage that will result in failure of downstream RNA transcription.
 - c. Cut the target bands (the sizes of the expected C, D, and E bands are 6,133 bp, 4,168 bp, and 1,636 bp, respectively) and extract the DNA fragment from the gel using a gel extraction kit (Omega) following the manufacturer's instructions.
 - d. At the final step, add 30 µl deionized water (pre-warmed at 65°C) onto the column and incubate at 37°C for 5 min.
 - e. Centrifuge at 13,000 × g for 1 min to elute the DNA.
 - f. Re-load the DNA elution onto the column and repeat Steps D2d and D2e to increase the recovery efficiency.
 - g. Measure quantity and quality of the recovered DNA using a NanoDrop.

Note: The recovery efficiency is approximately 30%.

3. *In vitro* ligation of fragments C, D, and E

- a. Set up a 10- μ l ligation reaction according to the components listed in **Table 8**. In this protocol, equal molar concentrations of fragments digested by BsAl are used.

Table 8. Fragments C, D, and E ligation reaction

Component	Volume (μ l)
10× T4 ligase buffer	1
Fragment C	310 ng
Fragment D	210 ng
Fragment E	55 ng
T4 ligase	1
Deionized water	Up to 10 μ l

- b. Incubate the reaction system at 4°C for 24 h. The ligation products (without purification) are utilized as a template for amplifying fragment C-D-E.

4. PCR amplification and purification of fragment C-D-E

- a. Set up a 50 μ l PCR reaction using the ligation products from Step D3b as the template. Prepare a 400 μ l reaction and aliquot it across eight 0.2-ml PCR tubes (50 μ l/tube). The components are shown in **Table 9**.

Table 9. PCR reaction for amplification of fragment C-D-E

Component	Volume (μ l)
5× PrimeSTAR® GXL buffer	10
2.5 mM dNTP	4
Forward primer THU3824 (10 μ M)	1
Reverse primer THU3825 (10 μ M)	1
Template (C-D-E ligation product)	10 ng
PrimeSTAR® GXL	1
Deionized water	Up to 50 μ l

- b. Incubate reactions in a thermal cycler according to the program shown in **Table 10**.

Table 10. Thermal cycling program for PCR amplification of fragment C-D-E

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	10 s	
Annealing and extension	68°C	10 min	30
Final extension	68°C	10 min	1
	16°C	hold	-

- c. Purify the PCR products by phenol-chloroform extraction as described in Step C4.

Note: Approximately 40 µg of C-D-E fragment can be recovered from the 400 µl PCR reaction solution.

- d. Load 2-µl onto a 1% agarose gel electrophoresis to check the quality of the purified PCR product, as shown in Figure 2B.

5. Fragments A-B and C-D-E digestion with Bsal

- a. Set up a 120-µl digestion reaction for fragments A-B and C-D-E as shown in Table 11.

Table 11. Digestion reaction for fragments A-B and C-D-E

Component	Fragment A-B	Fragment C-D-E
10× Cutsmart buffer	12 µl	12 µl
DNA fragment	40 µg	40 µg
Bsa I	6 µl	6 µl
Deionized water	Up to 120 µl	Up to 120 µl

- b. Incubate the digestion reaction at 37°C for 5 h.
- c. After digestion, the digestion products are separated by agarose electrophoresis and recovered by gel extraction using a kit as described in Step D2.
- d. At the final step of extraction, use 100 µl nuclease-free water for elution.

6. *In vitro* ligation of fragments A-B and C-D-E

- a. Set up a 100-µl ligation reaction, using equal molar concentrations of the fragments to assemble the full-length cDNA. The reaction components are listed in Table 12.

Table 12. Fragments A-B and C-D-E ligation reaction

Component	Volume (µl)
10× T4 ligase buffer	4
Fragment A-B	3.6 µg
Fragment C-D-E	2.3 µg
T4 ligase	3
Deionized water	Up to 100 µl

- b. Incubate the reaction at 4°C for 24 h.
- c. Add 1 µl of T4 ligase to the ligation reactions, mix well, and continue incubating at 4°C for another 24 h.
- d. Purify the full-length cDNA ligation products by phenol-chloroform extraction as described in Section C. At the final step of purification, dissolve the DNA pellet in 10 µl nuclease-free water. The purified DNA can be used immediately or stored at -20°C.
- e. Confirm the quality and size of the product by gel electrophoresis, loading 2 µl of purified ligation product onto a 1% agarose gel (Figure 2B).

E. Generation of viral genome-length RNA by *in vitro* transcription (IVT)

1. Preparation of N gene DNA.

In this protocol, N gene mRNA is electroporated into Caco-2-N cells along with genome-length viral RNA to increase trVLP production.

- a. Set up a 50 µl PCR reaction according to the PrimeSTAR® MAX instructions. The PCR reaction components are shown in **Table 13**.

Table 13. PCR system for amplification of N gene

Component	Volume (µl)
Forward primer THU2140 (10 µM)	1
Reverse primer THU2141 (10 µM)	1
Template (pLVX-N-Flag-IRES-mCherry)	30 ng
2× PrimeSTAR® Max	25
deionized water	Up to 50 µl

- b. Incubate reactions in a thermal cycler following the program in **Table 14**.

Table 14. Thermal cycling program for PCR amplification of N gene

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	15 s	
Annealing	55°C	15 s	30
Extension	72°C	25 s	
Final extension	68°C	5 min	1
	16°C	hold	-

- c. Check the quality of the PCR products by gel electrophoresis. Mix 2 µl PCR product with 6×DNA loading buffer and load the mixture onto a 1% agarose gel (**Figure 2C**).
- d. Recover N gene PCR products by phenol-chloroform extraction as described in Step C4. At the final step of purification, dissolve the DNA pellets in 30 µl nuclease-free water.

2. Set up a 30 μ l IVT reaction to generate full-length viral RNA and N gene mRNA using the Thermo mMESSAGE mMACHINE T7 transcription Kit, following the reaction setup shown in **Table 15**.

Table 15. IVT reaction for generating full-length viral RNA and N gene mRNA

Component	Volume (μ l)
2 \times NTP/CAP	15
GTP	4.5
10 \times reaction buffer	3
Template (FL-DNA ligation product)	1.5 μ g
T7 Enzyme mix	3
Nuclease-free Water	Up to 30 μ l

3. Incubate the IVT reaction at 32°C for 5 h.
 4. Add 1 μ l DNase to the reaction and incubate at 37°C for 15 min to digest the DNA template.
 5. Recover the viral RNA and N gene mRNA using lithium chloride precipitation following the manufacturer's instructions in the mMESSAGE mMACHINE® T7 transcription Kit. At the final step of the precipitation, dissolve the RNA pellet in 30 μ l nuclease-free water.
 6. Measure the concentration of RNA using a NanoDrop. Usually, approximately 30 μ g of viral RNA can be obtained.
 7. Load 1 μ g of RNA onto a 1% agarose gel to determine the quality of the genome-length RNA and N mRNA, as shown in **Figures 2D** and **2E**.
 8. Store the RNA at -80°C or use immediately.
- F. Electroporation of the genome-length viral RNA and N gene mRNA into Caco-2-N cell for trVLP production
1. Seed 3×10^6 Caco-2-N cells in a 10-cm dish 2 days before electroporation to make sure sufficient cells are present for electroporation.
 2. Prepare the following reagents and equipment before electroporation:
 - a. Cool the centrifuge to 4°C.
 - b. Pre-chill DPBS, Opti-MEM, and 4-mm cuvettes on ice.
 - c. Pre-warm a 10-cm dish containing 10 ml cell culture medium in a 37°C cell incubator.
 3. Harvest Caco-2-N cells
 - a. Add 2 ml of 0.25% trypsin/EDTA to the Caco-2-N cells in the 10-cm dish and incubate at 37°C for approximately 8 min to detach the cells.
 - b. Add 2 ml of cell culture medium to the plate to stop digestion and pipet to generate a single-cell suspension.
 4. Cell pretreatment and electroporation
 - a. Use a hemocytometer to determine the number of cells.
 - b. Transfer the appropriate volume of cell suspension containing 8×10^6 cells to a 15-ml tube

- and pellet the cells by centrifugation at $500 \times g$ for 5 min at 4°C .
- c. Discard the supernatant and wash cells by resuspending the pellet in 10 ml pre-chilled DPBS.
 - d. Precipitate cells by centrifugation at $500 \times g$ for 5 min at 4°C .
 - e. Remove the supernatant and resuspend the pellet in 400 μl Opti-MEM.
 - f. Add 20 μg genome-length viral RNA and 10 μg N gene mRNA to the cell suspension, pipetting up and down to mix thoroughly.
 - g. Transfer the entire cell mixture into the pre-chilled 4-mm cuvette, put the cuvette into the electroporation chamber, applying a single pulse (270 V at 950 μF) using the GenePulser apparatus (Bio-Rad).
 - h. After electroporation, immediately pipet the electroporated cells into the pre-warmed 10 cm dish containing 10 ml cell culture medium. Gently rock the plate and incubate at 37°C , 5% CO_2 .
 - i. Monitor GFP signal daily using a fluorescent microscope. Generally, the GFP signal can be observed 12 h post electroporation, as shown in **Figure 3A**.
5. Prepare Caco-2-N cells for *de novo* infection
Seed 1×10^5 Caco-2-N cells into a 24-well plate 24 h before *de novo* infection with the generated trVLPs, incubating at 37°C , 5% CO_2 .
 6. *De novo* infection
 - a. Check the GFP signal of the electroporated cells 24 h post electroporation
 - b. Collect 1 ml supernatant (containing trVLPs) from the electroporated cells (this will be considered P0).
 - c. Centrifuge the supernatant at $500 \times g$ for 5 min to pellet cell debris.
 - d. Make sure the confluence of the Caco-2-N cells in the 24-well plate seeded above in Step F5 is approximately 70-80%. Remove the cell culture medium and add the collected supernatant containing trVLPs into the well, and incubate at 37°C , 5% CO_2 .
 7. Monitor the GFP signal daily of the *de novo* infected cells (**Figure 3B**). Generally, the signal will be seen across the well 96 h post infection.
 8. From the *de novo* infected cells, harvest supernatant containing trVLPs 4 days post infection (defined as P1 virus), centrifuge at $500 \times g$ for 5 min at 4°C , and store as 1 ml aliquots at -80°C .
 9. To amplify the virus, add 50 μl of P1 virus onto Caco-2-N cells in a 10-cm dish (the confluence is 70-80%) and incubate at 37°C , 5% CO_2 .
 10. Monitor the GFP signal daily and harvest supernatants at 48 h post infection. Centrifuge at $500 \times g$ for 5 min at 4°C . The collected virus is defined as P2 and can be stored as 1 ml aliquots at -80°C until use.

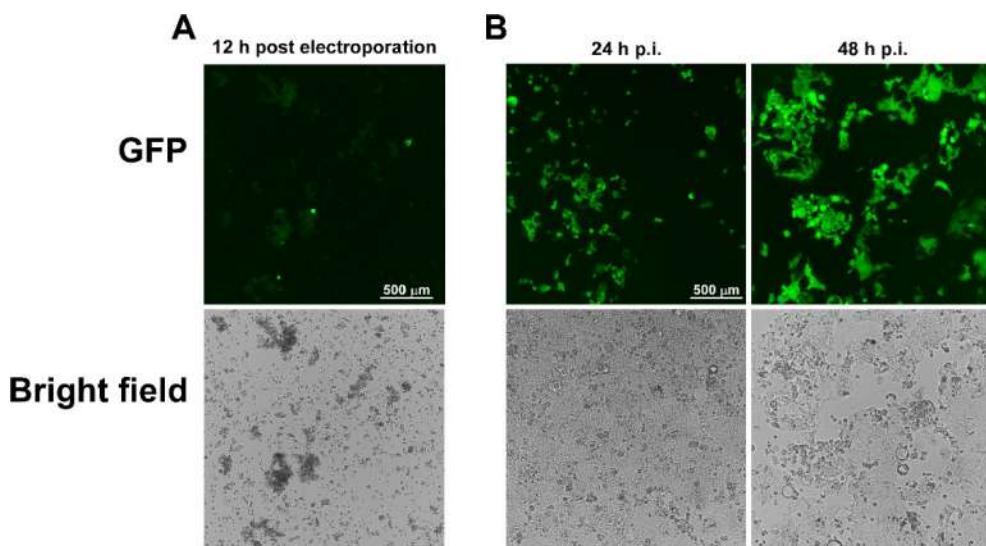


Figure 3. Fluorescence microscopy analysis of Caco-2-N cells infected with SARS-CoV-2 GFP/ΔN. (A) GFP and bright-field images of Caco-2-N cells at 12 h post electroporation. (B) Supernatants of Caco-2-N cells at 24 h post electroporation were collected and used to infect naive Caco-2-N cells in a 24-well plate. The GFP and bright-field images 24 h and 48 h post infection are shown.

G. RT-PCR verification of the GFP gene in SARS-CoV-2 GFP/ΔN trVLPs

To ensure the presence of the GFP gene in the SARS-CoV-2 GFP/ΔN trVLPs, viral RNA is extracted from the Caco-2-N cells infected by trVLPs and analyzed by RT-PCR (**Figure 4A**). A primer pair flanking the N region of ORF8 and the 3'UTR was designed for this purpose.

1. trVLP RNA extraction

- Prepare Caco-2-N cells in a 24-well plate one day before infection so that the confluence at time of infection is 70-80%.
- Infect cells by adding 50 μl trVLPs into the well and incubate at 37°C, 5% CO₂.
- 48 h post infection, discard the supernatant from the trVLP-infected cells and add 500 μl of TRIzol reagent into the well, pipetting up and down to make a single-cell suspension.
- Transfer the resultant cell lysate into a fresh 1.5 ml tube, add 100 μl chloroform, and vortex the tube or shake it by hand violently. Centrifuge at 13,000 × g for 15 min at 4°C.
- Carefully transfer 200 μl of the upper aqueous phase (containing RNA) to a fresh 1.5 ml tube, mix well with 200 μl isopropanol, and incubate at -20°C for 30 min.
- Centrifuge at 13,000 × g for 15 min at 4°C and remove the supernatant.
- Add 600 μl 75% ethanol to gently wash the pellet and centrifuge at 13,000 × g for 3 min at 4°C.
- Remove the supernatant and centrifuge the tube at 13,000 × g for 1 min at 4°C.
- Remove the supernatant and open the tube lid to dry the pellet thoroughly.
- Add 20 μl RNase-free H₂O to dissolve the RNA sample.

2. RT-PCR

- a. Use the Toyobo reverse transcription kit and the supplied random primers according to the manufacturer's direction to generate cDNA from the extracted viral RNA. A total of 10 μ l cDNA is obtained that can then be used immediately or stored at -20°C until use.
- b. Add 40 μ l H₂O to 10 μ l cDNA and mix well.
- c. Set up a 50 μ l PCR reaction using the cDNA as the template following the setup shown in **Table 16**.

Table 16. PCR reaction for verification of GFP gene

Component	Volume (μ l)
Forward primer THU2020 (10 μ M)	1
Reverse primer THU2195 (10 μ M)	1
Template (cDNA)	1
2 \times PrimeSTAR® Max	25
deionized water	Up to 50

- d. Incubate reactions in a thermal cycler following the program shown in **Table 17**.

Table 17. Thermal cycling program for PCR verification of GFP gene

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	15 s	
Annealing	55°C	15 s	30
Extension	72°C	20 s	
Final extension	68°C	5 min	1
	16°C	hold	-

- e. Examine the size of the PCR products by gel electrophoresis. Mix 5 μ l PCR product with 6 \times DNA loading buffer and load the mixture onto a 1% agarose gel (**Figure 4B**).

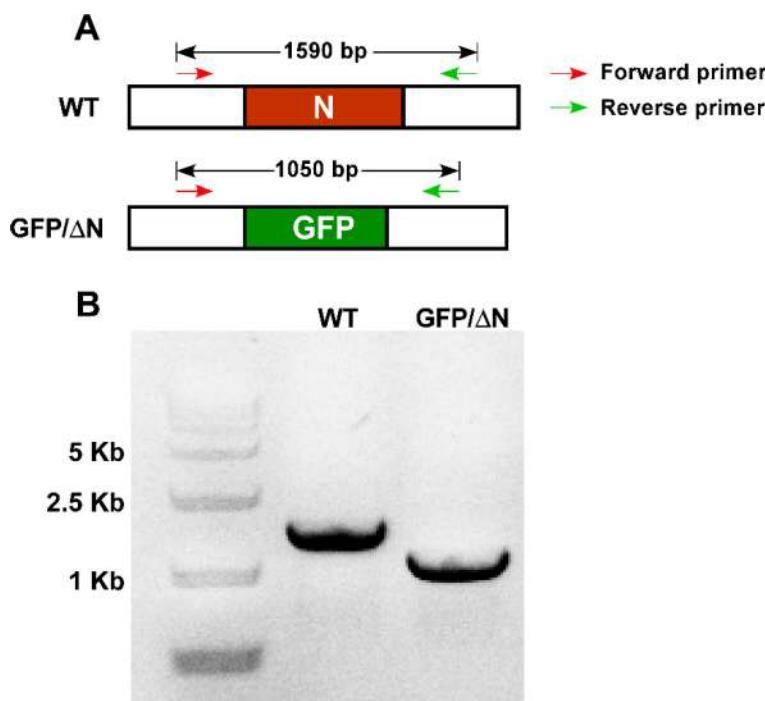


Figure 4. Analysis by RT-PCR of the GFP gene in trVLPs. RT-PCR validation of the GFP gene in SARS-CoV-2 GFP/ΔN trVLPs generated in Caco-2-N cells. The expected DNA size (A) and agarose electrophoresis analysis of the PCR products (B) are shown.

- H. trVLP titration using a tissue-culture infectious dose 50% (TCID₅₀) endpoint dilution assay
trVLP infectivity is quantified using a TCID₅₀ endpoint method (Lindenbach, 2009).
1. Seed 1.5×10^4 Caco-2-N cells in 100 μ l DMEM supplemented with 10% FBS per well in a 96-well plate one day before infection.
 2. Thaw the trVLPs stored at -80°C and serially dilute 1:10 using DMEM with 10% FBS (12 μ l trVLP sample into 108 μ l DMEM with 10% FBS). Mix by vortexing for 5 s and remove 12 μ l to add to another aliquot of 108 μ l DMEM with 10% FBS (1:100 dilution). Repeat the dilution series out to a 108-fold dilution.
 3. Add 100 μ l of the diluted virions to each well of the seeded Caco-2-N cells in the 96-well plate on top of the existing media (to make a total of 200 μ l per well), with eight replicates per dilution. Gently rock the plate to mix the virions well and incubate at 37°C, 5% CO₂.
 4. Check the GFP signal of each well 24 h post infection and count the number of GFP-positive wells for each concentration.
 5. Calculate the TCID₅₀ following the Reed & Muench method (Reed and Muench, 1938) using the equations described below:
 - a. $TCID_{50}/ml = (1 \text{ (ml)}/\text{the volume of original trVLPs (ml)}) \times 10^{\Delta} (\text{proportionate distance} + \text{Lower dilution})$.
 - b. Proportionate Distance = $(\text{Percentage next above } 50\% - 50\%) / (\text{Percentage next above } 50\% - \text{Percentage next below } 50\%)$.
 - c. Lower dilution = 10^{Δ} (dilution in which position is next above 50%).

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The original research article in which this protocol was used is Ju *et al.* (2021).

Competing interests

Q.D. and X.J. have filed a patent application on the use of the SARS-CoV-2 transcomplementation system and its use for anti-SARS-CoV-2 drug screening.

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A Standard Operative Procedure for Safe-handling of Remains and Wastes of COVID-19

Patients

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[Abstract] The ongoing coronavirus disease-2019 (COVID-19) pandemic has raised significant public health issues which need to be attended. To enable efficient handling of the situation and prevent the spread of the epidemic, healthcare professionals require various standard operating procedures (SOPs). Emerging evidence suggests high infectivity of the novel coronavirus strain—severe acute respiratory syndrome virus-2 (SARS-CoV-2)—causing COVID-19. The remains and wastes of COVID-19 patients can be a potential source of the virus exposure to the healthcare professionals if not handled with adequate precaution. Various institutions have issued SOPs in this regard, but a comprehensive approach is missing, which creates difficulty in interpretation and application of these SOPs. We have developed a comprehensive protocol for disposal of remains and wastes of the COVID-19 patients. We are following this protocol at our institution without any untoward event until date.

Keywords: COVID-19, SARS-CoV-2, Dead body, Waste management, Guidelines, Safe-disposal

[Background] Handling and disposal of the remains of the dead during the ongoing pandemic of coronavirus disease-2019 (COVID-19) are one of the most important and demanding aspects for the health care systems globally. COVID-19 is caused by a novel coronavirus strain (a positive sense single stranded RNA virus)—severe acute respiratory syndrome virus-2 (SARS-CoV-2). Emerging evidence suggests that SARS-CoV-2 has high infectivity (Atkinson and Petersen, 2020). The remains and wastes of COVID-19 patients can be a potential source of the virus exposure to the healthcare professionals, hence should be handled with adequate precaution. It is important as the health care system has to ensure that the dead body of a COVID-19 patient should not become a source of the infection, and demanding because of the varied religious beliefs and cultural practices that are associated with the way of disposing the dead body around the globe. In order to enable efficient handling of the remains and wastes of COVID-19 patients by the healthcare professionals, a stepwise protocol of standard operative procedures (SOPs) is essential. The emphasis should be on making the SOPs comprehensive

enough for easy interpretation and application.

A recent report (Sriwijitalai and Wiwanitkit, 2020) regarding the death of a forensic practitioner working in Bangkok, Thailand after contracting the virus from dead COVID-19 patient indicated the risk of transmission of the virus via dead bodies. This incidence has called for a protective protocol for handling the remains and wastes of the COVID-19 patients. Institutions like WHO (World Health Organization), CDC (Centers for Disease Control and Prevention, USA), Department of Health of different governments country wise (such as Ministry of Health and Family Welfare, MoHFW, Government of India; Department of Health, Hong Kong), etc. have laid down the protocols regarding the handling and disposal of the dead during COVID-19 (References 3-6). Though these protocols do elaborately explain the procedure for the same but are often difficult to interpret, and hence the application becomes inconvenient.

Here we have provided a set of instructions to be carried out in a sequential manner for the handling of remains and wastes of the COVID-19 patients providing additional details for precautions and safety measures, protecting gears and disinfecting materials. This protocol will help healthcare professionals taking quick on-spot decisions and avoiding exposure risks while handling the remains and wastes of COVID-19 patients.

Materials and Reagents

1. Liquid Soap
2. Water
3. 70% ethyl alcohol
4. EPA (the United States Environmental Protection Agency) registered disinfectant (Reference 7) (such as Sodium hypochlorite with a final concentration of 1%)

Equipment

1. Personal protective equipment (PPE) which includes
 - a. Nitrile gloves
 - b. Water-resistant gown/plastic apron over water repellent gown
 - c. N95 mask
 - d. Full-length cover shoes/boots
 - e. Goggles or face shield to protect eyes from the splashes
2. Measuring container
3. Plastic sheets for wrapping the body of the deceased
4. Zipper closed leak-proof transparent plastic bag (150 µm thick)
5. Tags
6. Gauge pieces
7. Spray bottle

8. Coloured Bins (Yellow, Red, White, Blue)
9. Non-chlorinated plastic bags with hazard sign (Figure 1) (Yellow, Red)



Figure 1. Bio-hazard Symbol (Reference 10)

10. Translucent, puncture, leak and tamper-proof White bags
11. Blue water proof cardboard boxes/containers
12. Incinerator
13. Industrial micro-wave/hydroclave/shredder

Procedure

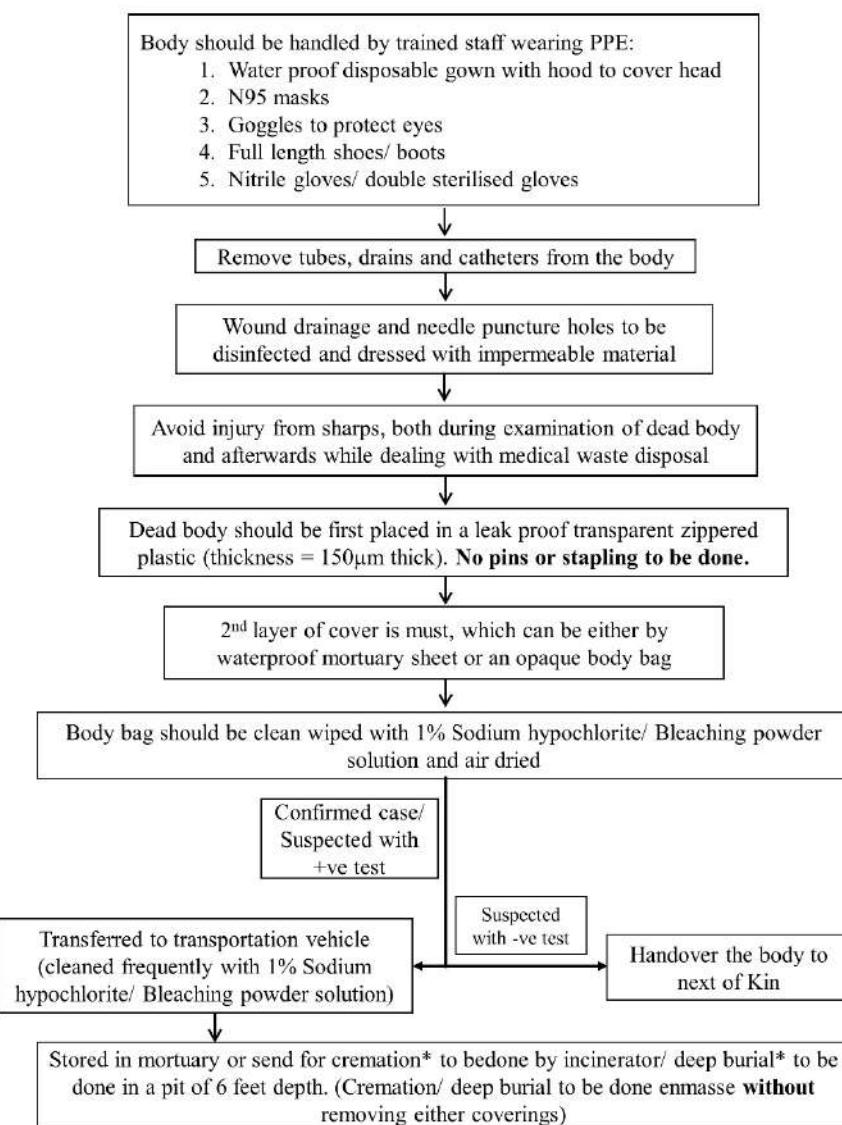
- A. Handling of the dead body by health care professionals (Tables 1-2) (Figure 2)

Table 1. The general recommendation for all persons handling the dead body of COVID-19 patients

1. The body should be handled by trained staffs while maintaining a high standard of personal hygiene such as washing hands with liquid soap and water or proper use of alcohol-based hand rub
2. Use of personal protective equipment (PPE) which includes:
 - a. Nitrile gloves
 - b. Water-resistant gown/ plastic apron over water repellent gown
 - c. Surgical mask
 - d. Full length cover shoes/ boots
 - e. Use goggles or face shield to protect eyes from splashes
3. Do not smoke, drink or eat. Do not touch the eyes, mouth or nose.
4. Avoid use and injury from needles and/or sharps during the examination of the dead body as well as later when dealing with the waste disposal and decontamination. All needle and sharps disposal should be done in the designated compartments.
5. Remove PPE after handling of the dead body and wash hands with liquid soap and water immediately.
6. In case of percutaneous injury or mucocutaneous exposure to blood or body fluids of the dead body, the injured or exposed areas should be washed with running water and soap for several minutes and inform your supervisor for prophylactic treatment and quarantine.
7. Clinical waste and its management are to be done according to the biomedical waste management criteria.
8. All the surfaces that came in contact with the patient, soiled linen and waste material, along with the reusable equipment should be disinfected using 70% ethyl alcohol for small areas—reusable dedicated equipment (e.g., thermometers) or Sodium hypochlorite at 1% for surface disinfection. Bodily fluids and blood stained wastes are to be first disinfected with Sodium hypochlorite at 1% and then disposed of as per the biomedical waste management rules.

Table 2. Group specific guidelines for disposal of the dead body of COVID-19 patients

	Health Care Professionals (HCP)	Mortuary Staff	Funeral Workers
Personnel protection and cleaning of the dead body	PPE should be properly put on by the staff handling the body	Autopsies or embalming of dead bodies of COVID-19 patients expose staff to the unwarranted risk and should generally be prohibited. In case autopsy is essential medico-legally, it should be done under strict precautionary measures as specified by concerned institutional guidelines. (Reference 4)	PPE should be properly put on by the staff handling the body
	Tagging the body for identification as infectious	Avoid direct contact with blood or body fluids from the dead body	
	Removal of tubes, drains and catheters from the body		
	Disinfect and dress the wound drainage and needle puncture holes with permeable material		
	The body should be cleaned and dried		
Dead body packaging for transport	The dead body should be first placed in a leak-proof transparent plastic bag (150 µm thick) and zipper closed. NO pins or stapling should be done.		The body should not be exposed out of the double layer covering for viewing
	A second layer of the cover is required, which can be either a wrapping with a mortuary sheet or placing the body in an opaque body bag.		
	The body bag should be cleaned with 1% Sodium Hypochlorite solution or bleach and then should be air dried.		
	Remove and dispose of the PPE in the container with lids marked for solid waste as per bio-medical waste disposal rules, and sanitize hands with water and soap.		Remove and dispose of the PPE in a container with lids marked for solid waste as per bio-medical waste disposal rules, and sanitize hands with water and soap.



*Cremation / deep burial of Covid 19 patients should be done by authorities in presence of Kin.

Clean all the surfaces that came in contact with dead body with 1% Sodium hypochlorite/ Bleaching powder solution)

Figure 2. Flowchart for steps of handling dead bodies of Confirmed/ Suspected COVID-19 patients

1. The body should be handled by trained and healthy (non-immunocompromised with no active respiratory infections) staffs (who have undergone evaluation of performance before executing the protocol) after maintaining a high standard of personal hygiene by washing hands with liquid soap and water or proper use of alcohol-based hand rub.
2. Donning of the personal protective equipment (PPE) is to be done in the following sequence (Reference 8):

- a. Perform hand hygiene by washing hands with soap and water for 60 s or rubbing hands with alcohol-based hand rub for 30 s.
- b. Put on the gown covering your feet and head.
- c. Put on shoe covers.
- d. Wash your hand with soap and water for 60 s or rubbing hands with alcohol-based hand rub for 30 s.
- e. Put on the mask.
- f. Put on eye protection gears.
- g. Put on the gloves.
3. Prepare 3 litre solution of EPA registered disinfectant (Reference 7) (such as 1% Sodium hypochlorite solution is prepared by mixing 2,700 ml of water with 300 ml of stock Sodium hypochlorite) with the help of measuring container and transfer the solution in a spray bottle.
4. Shift the body on a plastic sheet big enough to wrap the body (dimensions = 7 × 6 feet).
5. Avoid use and injury from needles and/ or sharps during the examination of the dead body. Remove the tubes, drains and catheters from the body of the deceased and close the puncture sites with the help of rubber or plastic adhesive tape in order to prevent any leakage. All needle and sharps disposal should be done in the designated compartments.
6. Pack the nostrils and oral orifice with gauge piece.
7. Spray the entire body with 1% Sodium hypochlorite and wait for it to dry.
8. Now, wrap the body in the plastic sheet sealing the same with adhesive plastic tape (remember, no stapling or pinning is to be done to avoid puncturing the sheet leading to leakage).
9. Spray the wrapped sheet with 1% Sodium hypochlorite and wait for it to dry.
10. Transfer the wrapped body in a zipper closed leak-proof transparent plastic bag (150 µm thick).
11. Spray the plastic bag with 1% Sodium hypochlorite and wait for it to dry.
12. Bodybag should be tagged, which should bear the name, age, sex, address and COVID-19 test status of the deceased.
13. Now, transfer the body with the bag on to the transportation vehicle for being shifted to the mortuary.
14. Doffing of the personal protective equipment (PPE) should be done in the following sequence (Reference 8) (Before doffing of the PPE, goggles or face shield, gloves, boots, water-resistant plastic gown must be sprayed with 1% sodium hypochlorite and allowed to dry):
 - a. Remove the gloves.
 - b. Remove the gown.
 - c. Perform hand hygiene by washing hands with soap and water for 60 s or rubbing hands with alcohol-based hand rub for 30 s.
 - d. Remove the eye protective gear.
 - e. Remove the mask.
 - f. Perform hand hygiene by washing hands with soap and water for 60 s or rubbing hands with alcohol-based hand rub for 30 s.

15. Dispose of the PPE in the container with lids marked for solid waste as per bio-medical waste disposal rules, and sanitize the hands with water and soap.

B. Handling of the body by mortuary staff (Table 2)

1. Staff at the mortuary should wear the disposable surgical cap, mask, gown, gloves and shoe cover while handling the dead body brought in a sterilized body bag.
2. The mortuary cooler cabin (maintained at 4°C), where the body (brought in the body bag) is to be kept, should also be tagged with identifying facts of the deceased to prevent any misadventure.
3. Autopsies or embalming of the dead bodies of COVID-19 patients may expose staff to unwarranted risk hence should be prohibited. In case autopsy is essential medico-legally, it should be done under strict precautionary measures (Reference 4; Hanley *et al.*, 2020).
4. While returning the body to next of the kin after test results (for viral RNA) are reported, one must ensure that the correct body is being handed over and to the authentic kin. Use of mask and gloves by the recipient of the body should be recommended.
5. If the test result is positive for the viral RNA (testing of each case should be done at least twice by the independent centres before declaring the patient positive), then the concerned authorities should be informed for maintaining the data as well as to ensure that the body bag remains unopened until being cremated or buried.

C. Handling of the body at Funeral homes/Cremation/Burial site (Table 2)

1. The transportation to the funeral place in case of the confirmed positive cases should be done under the supervision of government officials, and only trained staff should handle the dead body at the crematorium/or burial ground.
2. If the deceased is COVID-19 positive, then the body should be cremated or buried in the presence of government officials to ensure that the relatives have not opened the bag at any time during the transit or have handled the body without taking precautionary measures.
3. If the deceased is COVID-19 positive, the body bag can be opened just enough as to visualize the face to the relatives watching from at least 2-meter distance, and the staff should be wearing the full personal protective equipment (PPE) as being explained previously.
4. If the deceased is COVID-19 negative, then the body can be handled by the relative and the last rites can be performed as per will or religion of the deceased.
5. Dispose of the PPE in the container with lids marked for solid waste as per bio-medical waste disposal rules (References 10-12), and sanitize hands with water and soap.

D. Handling and disposal of the waste generated while caring a COVID-19 patient (Table 3)
(References 10-12)

Table 3. Biomedical wastes categories with their collection, packaging, treatment, processing and disposal options (References 10-12)

Colour coding (Type of bag/container)	Type of waste	Treatment disposal options
Yellow (Non-chlorinated color coded bags in colored bins)	Anatomical waste (Human tissue) Solid waste (Items contaminated with blood, and body fluids including cotton swabs, dressings, soiled plaster casts, disposable gowns, caps, bags containing residual or discarded blood and blood components, discarded linen, mattresses, beddings)	Incineration/deep burial* (deep burial is permitted only in rural or remote areas where there is no access to common bio-medical waste treatment facility)
Red (Non chlorinated plastic bags in coloured bins/ containers)	Contaminated Waste (Recyclable) Wastes generated from disposable items such as tubing, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and fixed needle syringes) and vaccutainers with their needles cut) and gloves	Autoclaving or micro-waving/ hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent to registered or authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible. Plastic waste should not be sent to landfill sites
White (Translucent, puncture, leak & tamper proof)	Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp object that may cause puncture and cuts. This includes both used, discarded and contaminated metal sharps	Autoclaving or Dry Heat Sterilization followed by shredding or mutilation or encapsulation in metal container or cement concrete; combination of shredding cum autoclaving; and sent for final disposal to iron foundries (having consent to operate from the State Pollution Control Boards or Pollution Control Committees) or sanitary landfill or designated concrete waste sharp pit
Blue (Water proof card board boxes/ containers)	Glassware waste - Broken or discarded and contaminated glass including medicine vials and ampoules	Disinfection (by soaking the washed glass waste after cleaning with detergent and Sodium Hypochlorite treatment) or through autoclaving or microwaving or hydroclaving and then sent for recycling.

1. The wastes are categorized and colour-coded into solid (yellow); liquid; contaminated waste (recyclable—red and non-recyclable—yellow); waste sharps—white/translucent and glassware wastes - blue.
2. The liquid waste is to be treated with EPA registered hospital disinfectant and discharged into drains.
3. Solid wastes comprising of human tissues, items contaminated with the blood of the patient, and body fluids including cotton swabs, dressings, soiled plaster casts, and disposable caps, masks, gowns, and shoe cover, bags containing residual or discarded blood and blood components, discarded linen, mattresses, beddings are to be kept in non-chlorinated yellow plastic bags inside yellow coloured bins. This categorical waste is to be disposed of by incineration (or deep burial is permitted only in rural or remote areas where there is no access to common bio-medical waste treatment facility).
4. Contaminated waste which is recyclable, i.e., waste generated from disposable items such as tubings, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and fixed needle syringes) and vacutainers with their needles cut and gloves should be kept in non-chlorinated red plastic bags inside the red coloured bins. These are disposed of by autoclaving or micro-waving or hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste should be sent to registered or authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible.
5. The translucent, puncture, leak & tamper-proof white coloured bags in white-coloured bins are used for keeping the contaminated sharps such as needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp object that may cause puncture and cuts. This includes used, discarded and contaminated metal sharps. These are disposed of by autoclaving or dry heat sterilization followed by shredding or mutilation or encapsulation in a metal container or cement concrete; a combination of shredding cum autoclaving; and are sent for the final disposal to iron foundries (having consent to operate from the State Pollution Control Boards or Pollution Control Committees) or sanitary landfill or designated concrete waste sharp pit.
6. Glassware waste such as broken or discarded and contaminated glass including medicine vials and ampoules are kept in blue coloured waterproof cardboard boxes/ containers. These are then disposed of by disinfection- either by soaking the washed glass waste after cleaning with EPA registered disinfectants (Reference 7) (such as detergent and 1% Sodium Hypochlorite treatment) or through autoclaving or microwaving or hydroclaving and then sent for recycling.
7. Plastic waste should not be sent to landfill sites.
8. All the containers and bins are to be marked with the universal biohazard symbol (Figure 1).

Acknowledgments

We consulted related guidelines issued by World Health Organization (WHO), Centers for Disease

Control and Prevention, USA, and Ministry of Health and Family Welfare (MoHFW), Government of India, and Government of Hong Kong for the preparation of this protocol. Authors express their sincere gratitude to above mentioned organizations.

Competing interests

Authors declare there are no competing interests. No funding was received for this work.

Ethics

Ethical issues related to the disposal of human remains and wastes in a hospital setting were considered during the preparation of this protocol.

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Identification and Quantitation of Neutrophil Extracellular Traps in Human Tissue Sections

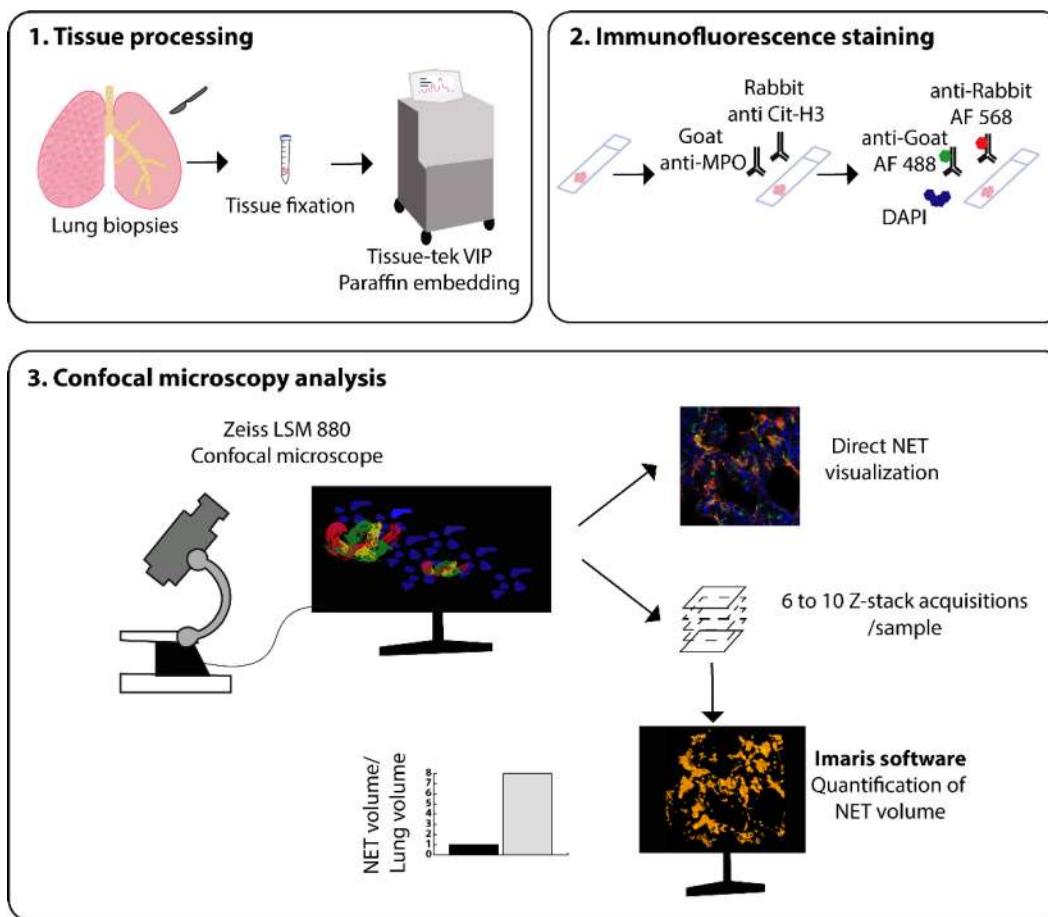
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[Abstract] Neutrophils are one of the first innate immune cells recruited to tissues during inflammation. An important function of neutrophils relies on their ability to release extracellular structures, known as Neutrophil Extracellular Traps or NETs, into their environment. Detecting such NETs in humans has often proven challenging for both biological fluids and tissues; however, this can be achieved by quantitating NET components (e.g., DNA or granule/histone proteins) or by directly visualizing them by microscopy, respectively. Direct visualization by confocal microscopy is preferably performed on formalin-fixed paraffin-embedded (FFPE) tissue sections stained with a fluorescent DNA dye and antibodies directed against myeloperoxidase (MPO) and citrullinated histone 3 (Cit-H3), two components of NETs, following paraffin removal, antigen retrieval, and permeabilization. NETs are defined as extracellular structures that stain double-positive for MPO and Cit-H3. Here, we propose a novel software-based objective method for NET volume quantitation in tissue sections based on the measurement of the volume of structures exhibiting co-localization of Cit-H3 and MPO outside the cell. Such a technique not only allows the unambiguous identification of NETs in tissue sections but also their quantitation and relationship with surrounding tissues.

Graphic abstract:



Graphical representation of the methodology used to stain and quantitate NETs in human lung tissue.

Keywords: Neutrophil, Neutrophil extracellular traps, Lung, Tissue sections, Confocal microscopy, NET quantitation

[Background] Neutrophils are the major innate immune cells in the blood (50-70% of white blood cells in humans) (Mestas and Hughes, 2004). These cells have a diameter of 5-7 μm and are characterized by a segmented nucleus, secretory vesicles in their cytoplasm, and a short lifespan (Borregaard, 2010). Neutrophils are produced in the bone marrow in response to G-CSF secretion under the control of key transcription factors such as PU.1, C/EBP α , GFI-1, and C/EBP ϵ (Hidalgo *et al.*, 2019). Neutrophils participate in pathogen destruction by releasing the cytotoxic contents of their secretory vesicles or by phagocytosis (Borregaard, 2010). Recently, several discoveries have shed light on neutrophil biology and function. First, a study using radiolabeling of neutrophils demonstrated an unexpected longer half-life of these cells, which can reach 5.4 days in humans and 18 hours in mice (Pillay *et al.*, 2010). Second, in 2004, Brinkmann and colleagues discovered a novel bactericidal action of neutrophils. Indeed, it was observed that the release of particular structures could trap bacteria and limit their dissemination throughout the organism (Brinkmann *et al.*, 2004). These new structures were named Neutrophil Extracellular Traps or NETs. NETs are web-like structures composed of extracellular free DNA

associated with a particular form of citrullinated histone 3 (Cit-H3), in which arginine residues have been replaced by citrulline, and various antimicrobial peptides from secretory vesicles, such as myeloperoxidase (MPO), neutrophil elastase (NE), or cathelicidins (Papayannopoulos, 2018). The release of these structures can be triggered by various stimuli derived from bacteria, viruses, parasites, and fungi, but also by immune complexes, crystals, pro-inflammatory cytokines, and chemokines (Papayannopoulos, 2018). Within neutrophils, the activation of pyruvate kinase C, formation of reactive oxygen species (ROS), and the activation of peptidyl arginase 4 and neutrophil elastase allow chromatin decondensation, nuclear membrane disruption, and NET release (Papayannopoulos, 2018). NETs can immobilize pathogens and prevent their dissemination but are also able to kill them directly (Brinkmann *et al.*, 2004; Papayannopoulos, 2018). Unfortunately, excessive NET release or inappropriate NET accumulation can trigger important tissue damage and lesions (Narasaraju *et al.*, 2011) or inadequate activation of immune cells, thereby promoting various immune-mediated disorders (Hakkim *et al.*, 2010; Radermecker *et al.*, 2019) or coagulation issues (Middleton *et al.*, 2020). Thus, NETs are increasingly implicated in various pathological processes, as evidenced by experimental work in mouse models (Radermecker *et al.*, 2019; Villanueva *et al.*, 2011). The investigation into their potential contributions to human disease is therefore of great interest. NET detection in humans is quite challenging and can be performed either indirectly in physiological fluids or directly in tissue sections. To date, NETs have been mainly measured in physiological fluids such as serum and bronchoalveolar lavage fluid (BALF) (Toussaint *et al.*, 2017; Middleton *et al.*, 2020). NET measurements in fluids rely on the detection of one or two of their components (extracellular DNA, citrullinated histone 3, or complexes of DNA/MPO or DNA/NE). Extracellular free DNA can be detected using DNA quantitation assays on serum or BALF supernatant (Toussaint *et al.*, 2017). Methods for the detection of Cit-H3 have been developed, including western blotting (Liu *et al.*, 2016; Thålin *et al.*, 2017). A more specific technique relies on the detection of complexes formed by DNA and one of the antimicrobial peptides released from secretory vesicles, like MPO or NE, by ELISA (Caudrillier *et al.*, 2012; Caldarone *et al.*, 2019). These techniques are rapid but lack specificity and do not provide any information about the location of NETs in the tissue. Here, we describe a protocol allowing the direct visualization and quantitation of NETs *in situ* by confocal microscopy. NETs are identified as extracellular structures exhibiting a co-localization of Cit-H3 and MPO, two important components of NETs. This technique is currently considered the gold standard of NET detection. Furthermore, we propose a novel software-based objective method to quantitate NETs in tissues based on the measurement of the volume of structures exhibiting a co-localization of Cit-H3 and MPO outside the cell (Radermecker *et al.*, 2019). This technique is specific, quantitative, and provides information about the location of NETs in tissues (Radermecker *et al.*, 2020). Identification of specific NET-rich areas in human tissues may promote a better understanding of their pathological roles in disease.

Materials and Reagents

1. Coverslips, 22 × 22 mm (Fisher Scientific, ThermoFisher Scientific, catalog number: 15727582)

2. Microscope slides, Superfrost (VWR, catalog number: 631-0113)
3. Pipette tips
4. Bovine serum albumin (Sigma-Aldrich, catalog number: 9048-46-8), stored at 4-8°C
5. Donkey serum (Sigma-Aldrich, catalog number: D9663), stored at -20°C
6. Goat anti-human/mouse myeloperoxidase antibody (Novus Biologicals, catalog number: AF3667), stored at -20°C
7. Rabbit anti-histone H3 (citrulline R2+R8+R17) antibody (Abcam, catalog number: ab5103), stored at -20°C
8. Donkey anti-goat IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488-conjugated (ThermoFisher Scientific, catalog number: A32814), stored at 4-8°C
9. Donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568-conjugated (ThermoFisher Scientific, catalog number: A10042), stored at 4-8°C
10. Neo Clear (Sigma-Aldrich, catalog number: 64741-65-7)
11. Absolute ethanol (Fisher Chemical, catalog number: E/0600DF/15)
12. Xylene (VWR, catalog number: 28973.363)
13. Ultrapure DNase/RNase-free distilled water (ThermoFisher Scientific, Invitrogen, catalog number: 10977049)
14. DAPI (Sigma-Aldrich, catalog number: 28718-90-3), stored at -20°C
15. ProLong Gold antifade mountant (ThermoFisher Scientific, catalog number: P10144), stored at -20°C
16. Goat IgG isotype control (ThermoFisher Scientific, catalog number: 31245), stored at 4-8°C
17. Rabbit IgG isotype control (ThermoFisher Scientific, catalog number: 31235), stored at 4-8°C
18. Formaldehyde (Sigma-Aldrich, catalog number: 47608)
19. HIER Tris-EDTA Buffer, pH 9.0 (10×) (Zytomed Systems, catalog number: ZUC029-500), stored at 4-8°C
20. Tween-20 (Fisher Scientific, Acros Organics, catalog number: 10491081), stored at room temperature (RT)
21. Triton X-100 (Sigma-Aldrich, catalog number: 9002-93-1), stored at room temperature (RT)
22. Antigen Retrieval Buffer (see Recipes)
23. Permeabilization Buffer (see Recipes)
24. Blocking Buffer (see Recipes)

Equipment

1. Tissue-Tek-VIP 5 (Sakura)
2. Rotary microtome (Leica)
3. Zeiss LSM 880 Airyscan Elyra s.1. confocal microscope (Zeiss)
4. StainTray slide staining system (Sigma-Aldrich, catalog number: Z670146)

5. Kartell PMPHellenthal staining jar (ThermoFisher Scientific, Fisher Scientific, catalog number: 10375681)
6. Portable steam sterilizer (Prestige Medical, 2100 classic)
7. Pipettes
8. Refrigerator (4°C)
9. Freezer (-20°C)
10. Chemical fume hood

Software

1. ImageJ (version 15.1n, NIH, <https://imagej.nih.gov/ij/download.html>)
2. Imaris (version 7.5.2, oxford instruments)
3. Zen black (Zeiss)

Procedure

A. Sample collection, fixation, and paraffin embedding

1. Collect lung post-mortem biopsies and fix them for three hours at RT in 4% formaldehyde.
2. After fixation, embed the biopsies in paraffin, following these three steps:
 - a. Dehydration: The fixation agent is eliminated by successive baths of increasing ethanol concentration: three times for 30 min in 70% ethanol, followed by three times for 30 min in 85% ethanol, and three times for 30 min in 100% ethanol.
 - b. Clearing: Immerse the tissues in a xylene bath three times for 20 min.
 - c. Embed in liquid paraffin at 59°C.

Note: These steps can be performed by an automated instrument like the “Tissue-Tek-VIP.”

3. Cut 4-μm-thick tissue sections using a rotary microtome and place on Superfrost slides.

B. Removal of paraffin

1. Rinse the slides in Neo Clear twice for 10 min.
2. Place the slides in a 100% ethanol bath for 5 min.
3. Place the slides in a 96% ethanol bath for 5 min.
4. Place the slides in an 80% ethanol bath for 5 min.
5. Place the slides in a 70% ethanol bath for 5 min.
6. Place the slides in a 50% ethanol bath for 5 min.
7. Rinse in ultrapure distilled water three times for one minute.
8. Rinse in 1× PBS.

C. Antigen retrieval

1. Place the slides in a vertical, plastic staining rack and add antigen retrieval buffer to totally

- immerse the paraffin-embedded samples (around 50 ml buffer for an 8-slide rack).
2. Fill the sterilizer with an adequate amount of distilled water.
 3. Place the rack in the sterilizer and close it.
 4. Allow the sterilizer to heat to 120-135°C, and incubate for 10-15 min.
 5. After 10-15 min, open the lid and allow the slides to cool to RT on the bench for approximately 20 min.
 6. Rinse twice with 1× PBS.
- D. Permeabilization
1. Immerse the slides in permeabilization buffer for 2 min.
 2. Rinse twice with 1× PBS.
- E. Blocking
1. Place the slides in the staining rack and incubate with blocking buffer for one hour at RT.
 2. Keep the excess blocking buffer at 4°C.
 3. During the blocking step, prepare the StainTray slide staining system. Place moistened paper in the tray.
 4. Rinse the slides once with 1× PBS.
- F. Primary staining
1. Prepare the primary staining mix. Use 80 µl staining mix for every 1 cm² of sample. Adapt the quantity of staining mix according to the surface area of the sample. Add goat anti-MPO at a 1/40 dilution and rabbit anti-histone 3 at a 1/100 dilution in blocking buffer.
 2. Place the slides in the staining system tray.
 3. Place the staining mix on the tissue sections to completely immerse them.
 4. Close the staining system and incubate at RT for one hour.
 5. Transfer the slides to a plastic rack.
 6. Rinse three times with 1× PBS.
- G. Secondary staining
1. Prepare the secondary staining mix. Add anti-goat Alexa Fluor 488 at a 1/200 dilution, anti-rabbit Alexa Fluor 568 at a 1/200 dilution, and DAPI at a 1/1,000 dilution in blocking buffer.
 2. Place the slides in the staining system tray.
 3. Place the staining mix on the tissue sections to completely immerse them.
 4. Close the staining system and incubate for two hours at RT.
 5. Transfer the slides to a plastic rack.
 6. Rinse three times with 1× PBS.
 7. Remove the moistened paper from the staining system tray.
 8. Dry the slides.

Important: Never touch the tissue sections.

9. From this point onward, work in a chemical fume hood to allow for optimal drying. Place a drop of ProLong Gold onto the tissue sections and add a coverslip. Allow the slides to dry overnight, protected from the light, but without totally closing the staining system to facilitate air circulation. In some experiments using other mounting media, we observed a rapid loss of the Cit-H3 staining. Since ProLong Gold is described to protect fluorescent dyes from fading and photobleaching, we chose this mounting medium.
10. Analyze the slides and acquire images using a confocal microscope within two days of staining.

H. Controls

You can perform two types of negative control. You can perform primary staining with isotype control antibodies, *i.e.*, goat IgG and rabbit IgG isotype control antibodies instead of anti-MPO and anti-citrullinated Histone 3, at similar concentrations. Alternatively, you can replace the primary staining with an incubation with sera from the host species in which the primary antibodies were produced (*i.e.*, rabbit and goat serum). In both cases, the secondary staining remains the same.

Data analysis

A. Image acquisition

Images are acquired using a confocal microscope (here, a Zeiss LSM 880). Select the appropriate lasers; in this case, 488 nm (MPO), 561 nm (Cit-H3), and 405 nm (DAPI). Adjust the laser voltages to identify stained structures. To ensure the specificity of your staining, compare positive samples with control samples. Once the lasers have been set up, be sure to always use the same settings for all the slides that you want to analyze and compare. NETs are identified as extracellular structures that stain positive for MPO and Cit-H3. DAPI staining is not always observed in NETs, as NETs are composed of decondensed chromatin (Figure 1). To further quantitate the NETs, we performed Z-stack acquisition of 6-10 random fields on each section at 20 \times magnification.

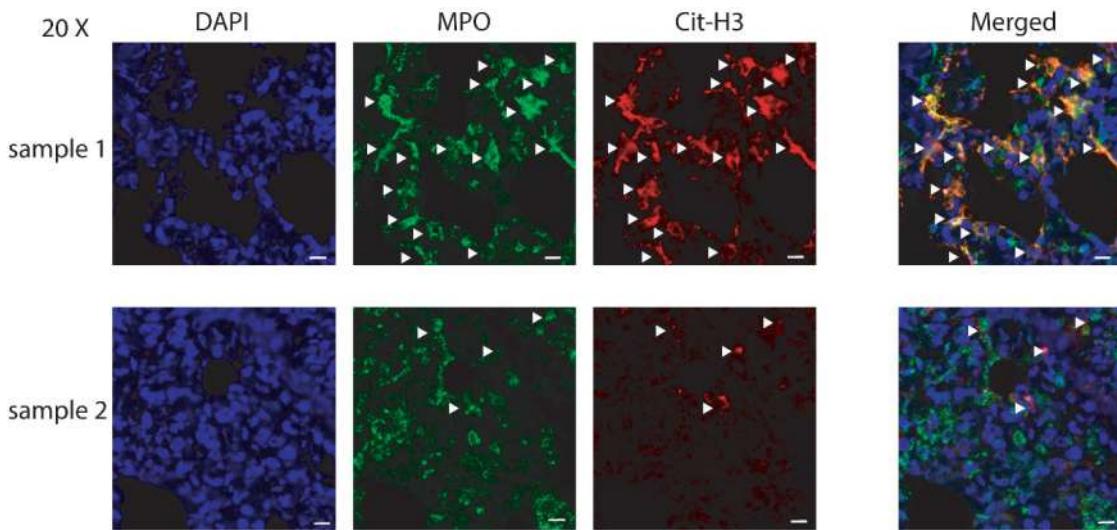


Figure 1. Representative images of lung NETs. NETs are identified as extracellular structures where MPO (AF-488, green) and Cit-H3 (AF568, red) co-localize (shown by white arrows). Co-localization with DAPI could not always be observed. Images were acquired at 20 \times magnification. Scale bars, 10 μ m.

B. Quantitation of NET volume

To quantitate NETs in lung tissue sections (Figure 2), we analyzed 6-10 (20 \times magnification) per section, and one section per patient (Radermecker *et al.*, 2020). We acquired Z-stack images using the Imaris software. We performed a three-dimensional reconstruction of structures that stained double-positive for Cit-H3 (red) and MPO (green) (Figure 2), and Imaris provided quantitation of the volume of these structures. The co-localization method uses three Imaris script/macro tools (Table 1). The first script processes all the .ims files in a folder, then thresholds the red Cit-H3 and green MPO staining. During this step, we used a threshold to separate voxels from background, and voxels from staining, in each separate channel. We defined true staining as all the staining above 10 voxels. Then, the thresholding method replaced each pixel in an image with a black pixel, if the image intensity was less than the threshold previously fixed (10 voxels), or a white pixel, if the image intensity was greater than that threshold. Subsequently, we created a new channel, *i.e.*, the intersection between the red and green thresholds, where all the voxels have a co-localization equal to 1. The second script then measured the volume of the intersection between the red and green thresholds. Finally, the third script measured the total volume of the image and created an Excel .xlsx file containing all the measurements. Finally, we divided the volume obtained (volume of the intersection of red and green channels) by the total volume of lung tissue analyzed in each sample to yield the cubic micrometers of NETs per cubic micrometer of lung tissue. All the procedures for quantitation are represented in Figure 3.

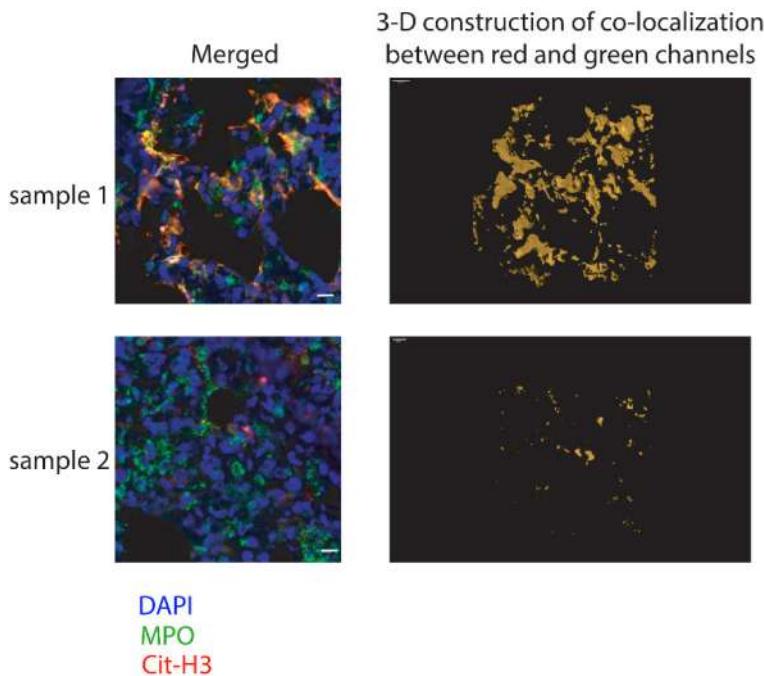


Figure 2. Example of a 3D construction of volumes, where co-localization of MPO and Cit-H3 is present. Confocal microscopy staining (left) and three-dimensional modeling (right) of Cit-H3+MPO+ NETs. Scale bars, 10 μ m.

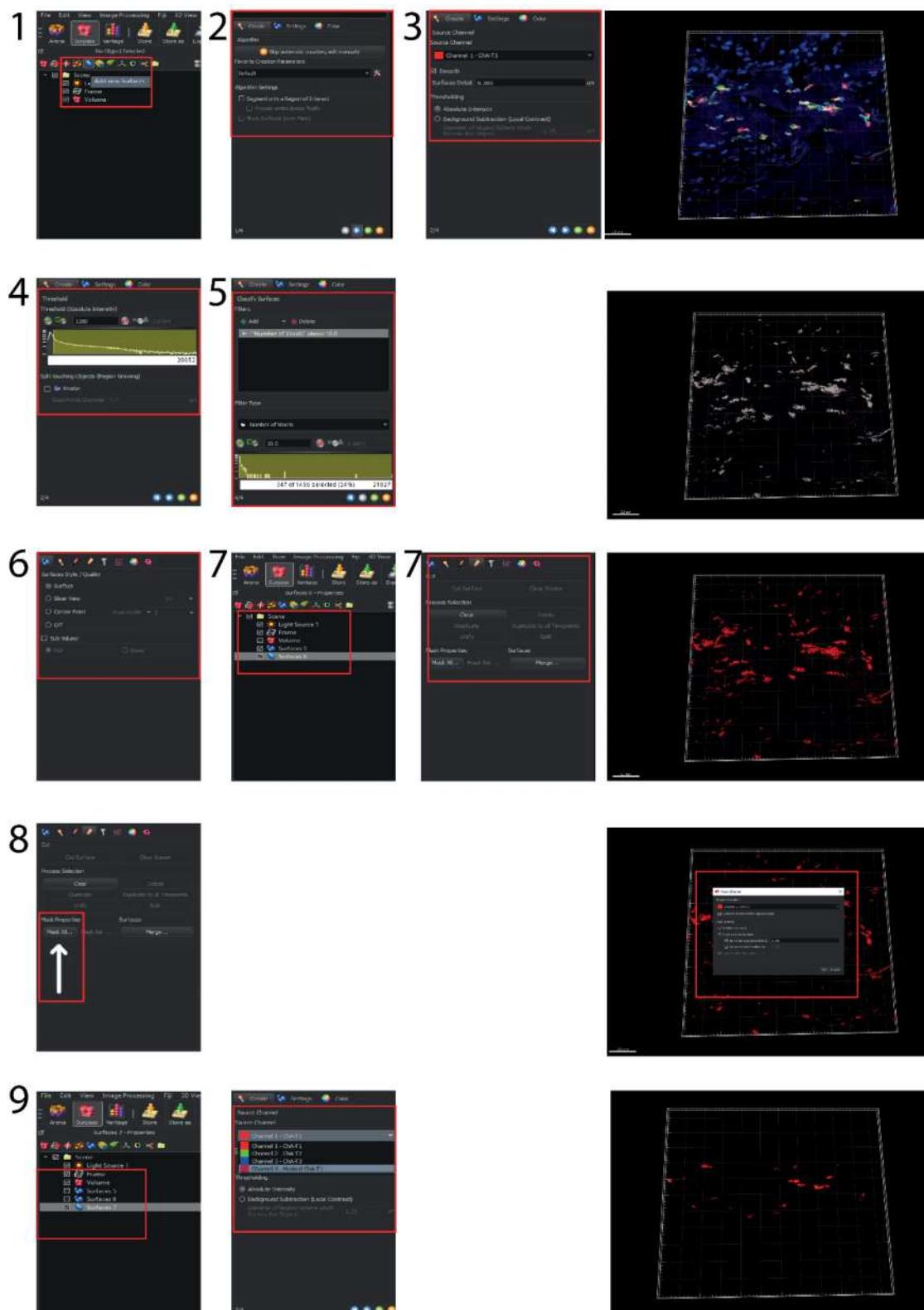


Figure 3. Procedure for the 3D reconstruction of volumes, where co-localization of MPO and Cit-H3 is present on Imaris. 1) Add a new red surface; 2) Initiate; 3) Choose the red channel, smooth 0.2, absolute intensity; 4) Set the threshold to 1280; 5) Classify the surface above 10; 6) Validate the surface; 7) Repeat steps 2-6 with the green channel; 8) Select channel green > cut >

Mask all > Channel 1 and duplicate the channel before applying mask > constant outside to 0; 9)
Repeat steps 2-6 with Mask of red channel.

Table 1. Scripts used for the quantitation of NET volume

Green algorithm	Red algorithm
Enable Region Of Interest = false	Enable Region Of Interest = false
Enable Region Growing = false	Enable Region Growing = false
Enable Tracking = false	Enable Tracking = false
[Source Channel]	[Source Channel]
Source Channel Index = 4	Source Channel Index = 1
Enable Smooth = true	Enable Smooth = true
Surface Grain Size = 0.200 µm	Surface Grain Size = 0.200 µm
Enable Eliminate Background = false	Enable Eliminate Background = false
Diameter Of Largest Sphere = 1.00 µm	Diameter Of Largest Sphere = 1.00 µm
[Threshold]	[Threshold]
Enable Automatic Threshold = false	Enable Automatic Threshold = false
Manual Threshold Value = 1200	Manual Threshold Value = 1030
Active Threshold = true	Active Threshold = true
Enable Automatic Threshold B = true	Enable Automatic Threshold B = true
Manual Threshold Value B = 10964.9	Manual Threshold Value B = 6835.53
Active Threshold B = false	Active Threshold B = false
[Classify Surfaces]	[Classify Surfaces]
"Number of Voxels" above 10.0	"Number of Voxels" above 10.0

C. Statistical analysis

We represented the data as the mean for each section and analyzed it for statistical significance using a non-parametric Mann-Whitney U test on the mean values. *P* values < 0.05 were considered statistically significant.

Notes

- a) While the current protocol has been mainly employed in human lung formalin-fixed paraffin-embedded (FFPE) sections, it has also been applied to human liver, pancreas, kidney, and heart FFPE sections (Radermecker *et al.*, 2020). While we did not detect NET structures in those organs of severe Covid-19 patients, neither did we detect non-specific staining, suggesting that the protocol could also be applied to these organs.
- b) Of note, MPO can also be expressed by other phagocytes, such as human macrophages or eosinophils, which may also form extracellular traps and stain for Cit-H3; therefore, the present

protocol would be more specific for neutrophils and NETs if an additional stain directed against a neutrophil marker, such as Ly-6G, was used. From our experience, anti-Ly-6G antibodies do not work properly on FFPE sections, and some clones also detect Ly-6C, which does not help to solve the specificity issue. In laboratory mice, selective depletion of neutrophils, such as an anti-Ly-6G-mediated depletion, can be used to confirm the specificity of the method, as performed previously (Radermecker *et al.*, 2019). Indeed, NETs were shown to be present in lungs from neutrophil-sufficient mice exposed intranasally to a low dose of lipopolysaccharide, but NETs were absent in neutrophil-depleted mice (Radermecker *et al.*, 2019).

Recipes

1. Antigen Retrieval Buffer

Make a 1/10 dilution of HIER Tris-EDTA Buffer pH 9.0 (10×) in distilled water. Add Tween-20 at a final concentration of 0.05%.

2. Permeabilization Buffer

Add Triton X-100 to PBS at a final concentration of 0.5%.

3. Blocking Buffer

Add BSA at a final concentration of 2% and donkey serum at a final concentration of 2% in PBS.

Acknowledgments

The immunofluorescence protocol was adapted from an article by Toussaint and colleagues (Toussaint *et al.*, 2017). The quantitation method is described in Radermecker *et al.* (2019 and 2020).

Competing interests

The authors declare no competing interests.

Ethics

The use of human specimens was approved in 2020 by the Ethics Review Board of the University Hospital of Liege (ref 2020/119).

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A One-enzyme RT-qPCR Assay for SARS-CoV-2, and Procedures for Reagent Production

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[Abstract] Given the scale of the ongoing COVID-19 pandemic, the need for reliable, scalable testing, and the likelihood of reagent shortages, especially in resource-poor settings, we have developed an RT-qPCR assay that relies on an alternative to conventional viral reverse transcriptases, a thermostable reverse transcriptase/DNA polymerase (RTX) (Ellefson *et al.*, 2016). Here we show that RTX performs comparably to the other assays sanctioned by the CDC and validated in kit format. We demonstrate two modes of RTX use – (i) dye-based RT-qPCR assays that require only RTX polymerase, and (ii) TaqMan RT-qPCR assays that use a combination of RTX and Taq DNA polymerases (as the RTX exonuclease does not degrade a TaqMan probe). We also provide straightforward recipes for the purification of this alternative reagent RTX. We anticipate that in low resource or point-of-need settings researchers could obtain the available constructs and begin to develop their own assays, within whatever regulatory framework exists for them.

Keywords: Coronavirus, SARS-CoV-2, Quantitative RT-PCR, Reverse transcriptase, TaqMan RT-qPCR, Nucleic acid diagnostics

[Background] While various virus detection methods have been implemented for the detection of SARS-CoV-2 infection, including a variety of molecular diagnostics and immunodiagnostic tests, the Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) remains the primary and most sensitive test for SARS-CoV-2 detection (D'Cruz *et al.*, 2020; Tang *et al.*, 2020). The primacy of RT-qPCR is in large measure because antibody-based tests as well as rapid nucleic acid diagnostic platforms, such as Abbott IDNow, often suffer from poor sensitivity, especially during early infection when viral loads are generally low in patients (Basu *et al.*, 2020; D'Cruz *et al.*, 2020). Given the importance of the early diagnosis in containing COVID-19 outbreak (Peck, 2020), the need for a rapid and scalable RT-qPCR setup is imminent. Unfortunately, there are increasing shortages of a wide variety of reagents necessary to scale RT-qPCR-based tests (Pettit *et al.*, 2020). The main manufacturers of PCR platforms cannot scale-up the production of tests in sufficient quantities to supply resource-poor settings. Even were resource-poor settings to attempt to develop their own solutions, researchers, clinicians, and public health officials often lack the necessary reagents, including enzymes, to develop testing programs (Kavanagh *et al.*, 2020).

Herein, we layout protocols for dye-based and TaqMan probe-based RT-qPCR assays for CDC-designed SARS-CoV-2 N gene tests (<https://www.fda.gov/media/134922/download>). We detail a one-enzyme RTX-based RT-qPCR protocol, as well as a two-enzyme RTX/Taq DNA polymerase-based RT-

qPCR protocol. While the dye-based one enzyme and the TaqMan probe-based two enzyme RT-qPCR both exhibit comparable performances in the detection of viral RNA, the TaqMan probe-based assay exhibited lower background. In these, we compare our RTX enzyme preparations, for which we provide a detailed purification protocol, with commercially available reagents.

Our RTX-mediated protocols are as sensitive as those that rely on enzyme combinations or commercial enzymes, and can expedite SARS-CoV-2 tests by reducing the number of kits or key enzymes that must be purchased. These protocols can also form the basis for further assay simplification and democratization via the use of so-called ‘cellular reagents’: polymerase-overexpressing cells that can be directly added to molecular diagnostics assays without loss of sensitivity or specificity (Bhadra *et al.*, 2018).

Materials and Reagents

A. For RTX polymerase-based RT-qPCR analysis

1. Barrier tips for pipets
2. Tris-HCl pH7.5 and 8.0 (Sigma-Aldrich, catalog number: T1503)
3. $(\text{NH}_4)_2\text{SO}_4$ (Sigma-Aldrich, catalog number: A4413)
4. KCl (Sigma-Aldrich, catalog number: P3911)
5. MgSO_4 (Sigma-Aldrich, catalog number: M7506)
6. D-glucose (Sigma-Aldrich, catalog number: 47829)
7. 1% Triton® X-100 (New England Biolabs, B9004S), store at -20°C
8. Forward PCR primers (Integrated DNA Technologies, **Table 1**) (Note a), store at -20°C, 100 µM Stock
9. Reverse PCR primers (Integrated DNA Technologies, **Table 1**) (Note a), store at -20°C, 100 µM Stock
10. TaqMan probes (Integrated DNA Technologies, **Table 1**) (Note a), store at -20°C
11. 20× EvaGreen solution (Biotium, #31000), store at 4°C
12. 4 mM deoxyribonucleotides (dNTP) (New England Biolabs, N0446S), store at -20°C
13. 5 M Betaine (Sigma-Aldrich, B0300), store at 4°C
14. 20 U/µl SUPERase·In RNase Inhibitor (Thermo Fisher Scientific, AM2694), store at -20°C
15. 5 U/µl Taq DNA polymerase (New England Biolabs, M0267L), store at -20°C
16. 0.2 mg/ml RTX polymerase (Note b) (purified by Ellington lab), store at -20°C
17. Nuclease-free water (store at room temperature)
18. qPCR tubes or plates with optical covers (Note c) (Roche, 04729692001), store at room temperature
19. Cold-block or ice
20. 10× RTX buffer (see Recipes)
21. 10× ThermoPol buffer (see Recipes)

Notes:

- a. Individual primer and probe stocks or pre-made primer-probe mixes for the CDC N1, N2, and N3 assays (**Table 1**) available from Integrated DNA Technologies may be used.
- b. RTX polymerase with proofreading capability (RTX), and RTX polymerase without proofreading capability (RTX Exo-) have been compared.
- c. LightCycler 96 real-time PCR machine and 96-well plates with optical plastic film cover designed for use with LightCycler platform were used for these experiments.

Table 1. CDC TaqMan RT-qPCR primers and probes for SARS-CoV-2^a

Name	Description	Sequence ^b
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
2019-nCoV_N1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'
2019-nCoV_N2-P	2019-nCoV_N2 Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'

^aPrimer and probe sequences adapted from <https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html>; last accessed on May 6th, 2020. According to the CDC, oligonucleotide sequences are subject to future changes as the 2019-Novel Coronavirus evolves. Refer to the CDC website for the latest updates.

^bFAM: 6-carboxyfluorescein; BHQ-1: Black Hole Quencher 1

B. For purification of RTX DNA polymerase

1. Sterile culture tubes and flasks
2. Centrifugation tubes (Nalgene, catalog number: 3115-0050)
3. Sterile syringe filter 0.2 µm (PES, VWR, catalog number: 28145-501)
4. RTX or RTX Exo- polymerase expression plasmid (Addgene, Plasmid #102787 and #102786)
5. Competent *E. coli* T7 RNA polymerase-based protein expression strain, such as BL21 (DE3) (New England Biolabs, C2527H), store at -80°C
6. Antibiotics such as ampicillin or carbenicillin (Goldbio, catalog number: 4800-94-6)
7. Superior Broth™ (Athena Enzyme Systems™, catalog number: 0105), store at room temperature
8. 1 M Isopropyl-β-D-thiogalactoside (IPTG) (Sigma Aldrich), store at -20°C
9. Ni-NTA agarose (Thermo Fisher Scientific, catalog number: 88223), store at 4°C

10. Disposable protein purification columns (Thermo Fisher Scientific, catalog number: 29924)
11. 20,000 MWCO Dialysis cassette (Thermo Fisher Scientific, catalog number: 66012)
12. HiTrap™ Heparin HP column (GE Healthcare, catalog number: 17-0406-01)
13. Bis-Tris mini protein gel (Thermo Fisher, catalog number: NP0321BOX)
14. EDTA-free protease inhibitor tablet (Thermo Scientific, catalog number: A32965)

Note: All chemicals were molecular biology or analytical grade and purchased from Sigma-Aldrich (see below for catalog number) unless otherwise indicated.

15. NaCl (Sigma-Aldrich, catalog number: S7653)
16. Imidazole (Sigma-Aldrich, catalog number: I5513)
17. Igepal CO-630 (Sigma-Aldrich, catalog number: 542334)
18. MgSO₄ (Sigma-Aldrich, catalog number: M7506)
19. HEW Lysozyme (Sigma-Aldrich, catalog number: L6876)
20. Tris (Sigma-Aldrich, catalog number: T1503)
21. DTT (Sigma-Aldrich, catalog number: D9779)
22. KCl (Sigma-Aldrich, catalog number: P3911)
23. Tween-20 (Sigma-Aldrich, catalog number: P9416)
24. Purification buffers for RTX DNA polymerase purification, store at 4 °C (see Recipes)
 - Resuspension Buffer
 - Equilibration Buffer
 - Lysis Buffer
 - Wash Buffer
 - Elution Buffer
 - Heparin Buffer A
 - Heparin Buffer B

25. Dialysis buffers for dialysis of RTX DNA polymerase, store at 4°C (see Recipes)
 - Ni-NTA Dialysis Buffer
 - Heparin Dialysis Buffer
 - Final Dialysis Buffer

Equipment

1. Magnetic stir bar
2. Magnetic stirrer
3. Real-time PCR machine

Note: LightCycler96 real-time PCR machine from Roche was used for these experiments.

4. Refrigerated shaker incubator (New Brunswick Scientific, model: Innova44)
5. Sonicator (Fisher Scientific, Sonic Dismembrator Model 500)
6. Centrifuge (Beckman Coulter, model: Avanti JXN-26, Fixed angle)

7. Thermomixer (Eppendorf, model: Thermomixer C)
8. Fast protein liquid chromatography (FPLC) machine (GE Healthcare, model: AKTA pure)
9. Fraction collector (GE Healthcare, model: Fraction Collector F9-R)

Software

1. LightCycler96 software (Roche; available for download from https://lifescience.roche.com/en_us/brands/realtime-pcr-overview.html#software)

Procedure

A. Dye-based RT-qPCR reaction set up using only RTX DNA polymerase

1. Set up EvaGreen dye-containing RT-qPCR reactions for CDC SARS-CoV-2 N1, N2, and N3 assays as shown in **Table 2**. The three assays target three different regions within the nucleocapsid phosphoprotein (N) gene near the 3'-end of the ~30 kb viral genome. In particular, N1 primers (**Table 1**) amplify a 72 nt long region from position 28287 to 28358 of the genome (GenBank Accession No. MN985325.1). The N2 primers produce a 67 bp amplicon from the genomic position 29164 to 29230. Meanwhile, the N3 primers amplify a 72 nt long region from genomic position 28681 to 28752. RNA isolation should be performed before RT-qPCR.

Table 2. EvaGreen RT-qPCR reaction mix using only RTX DNA polymerase

	Volume (μl)	Final concentration
RNA sample ^a	5	
5 μM Forward PCR primer	2.5	500 nM
5 μM Reverse PCR primer	2.5	500 nM
20× EvaGreen	1.25	1×
10× RTX buffer	2.5	1×
4 mM dNTP	2.5	400 μM
20 U/μl SUPERase·In RNase Inhibitor	1	0.8 U/μl
RTX or RTX Exo- 0.2 mg/ml	0.5	4 ng/μl
5M Betaine	2.5	0.5 M
Water	4.75	
Total reaction volume	25	

^aFull length SARS-CoV-2 or MERS-CoV N synthetic RNAs were used in these studies. The RNA copies per reaction (100 to 100,000 copies) are indicated in the relevant figures. Patient saliva is reported to have median SARS-CoV-2 viral loads of 3.3×10^6 copies/ml (To et al., 2020).

2. Assemble all reaction mixes on a cold-block and directly transfer to a real-time PCR machine programmed to cycle through the steps depicted in **Table 3**. Include Melt curve analysis steps

after qPCR amplification in order to distinguish target amplicons from spurious products.

Table 3. RTX polymerase-based EvaGreen RT-qPCR cycling conditions

Step	Temperature	Duration	Signal acquisition
1 ^a	60°C	30 min	None
2 ^a	95°C	10 min	None
55 cycles of Steps 3 and 4			
3	95°C	15 s	None
4 ^b	55°C	30 s	Single
Melt curve analysis in Steps 5, 6, and 7			
5	95°C	10 s	None
6	65°C	60 s	None
7	97°C	1 s	Continuous ^c

^aAssay time may be reduced by shortening the reverse transcription step (step 1) to 15 min and the following denaturation step (step 2) to 2 min.

^bSignal acquisition occurs in the FAM (fluorescein) channel during steps 4 and 7.

^c20 measurements/°C temperature change

B. RTX polymerase-based TaqMan RT-qPCR reaction set up

1. In order to perform TaqMan RT-qPCR we developed a one-pot two enzyme system comprising RTX and Taq DNA polymerases. Set up RTX-based TaqMan RT-qPCR reactions for CDC SARS-CoV-2 N1, N2, and N3 assays as shown in **Table 4**.
2. Assemble all reaction mixes on a cold-block and directly transfer to a real-time PCR machine programmed to cycle through the steps depicted in **Table 5**.

Table 4. RTX polymerase-based TaqMan RT-qPCR reaction mix

	Volume (μl)	Final concentration
RNA sample	5	
6.7 μM Forward PCR primer	1.5	402 nM
6.7 μM Reverse PCR primer	1.5	402 nM
1.7 μM TaqMan probe	1.5	102 nM
10× Assay buffer ^a	2.5	1×
4 mM dNTP	2.5	400 μM
20 U/μl SUPERase-In RNase Inhibitor	1	0.8 U/μl
5 U/μl Taq DNA polymerase	0.5	0.1 U/μl
RTX or RTX Exo- 0.2 mg/ml or water ^b	0.5	4 ng/μl
5M Betaine	2.5	0.5 M
Water	6	
Total reaction volume	25	

^aAssay buffer: Duplicate TaqMan RT-qPCR reactions were performed either in 1× ThermoPol

buffer or in 1× RTX buffer as indicated to determine optimum buffer conditions.

^bControl reactions lacking RTX polymerase were set up using the same recipe except RTX was replaced with water.

Table 5. RTX polymerase-based TaqMan RT-qPCR cycling conditions

Step 1 ^a	60°C	30 min
Step 2 ^a	95°C	10 min
55 cycles of Steps 3 and 4		
Step 3	95°C	15 sec
Step 4 ^b	55°C	30 sec

^aAssay time may be reduced by shortening the reverse transcription step (step 1) to 15 min and the following denaturation step (step 2) to 2 min without compromising results.

^bSignal acquisition occurs in the FAM (fluorescein) channel during step 4.

C. SARS-CoV-2 RT-qPCR using commercial TaqPath™ 1-Step RT-qPCR Master Mix, CG

1. To perform CDC N1, N2, and N3 SARS-CoV-2 TaqMan RT-qPCR assays using commercial assay master mix, set up reactions using TaqPath™ 1-Step RT-qPCR Master Mix, CG (Thermo Fisher Scientific, Waltham, MA, USA) as shown in **Table 6**.
2. Assemble all reactions on a cold-block and then incubate at room temperature (25 °C) for 2 min prior to cycling on a real-time PCR machine using the steps indicated in **Table 7**.

Table 6. CDC N1, N2, N3 TaqMan RT-qPCR set up using commercial RT-qPCR master mix

	Volume (μl)
RNA sample	5
6.7 μM Forward PCR primer	1.5
6.7 μM Reverse PCR primer	1.5
1.7 μM TaqMan probe	1.5
4× TaqPath™ master mix	5
Water	5.5
Total reaction volume	20

Table 7. TaqPath™ TaqMan RT-qPCR cycling conditions

Step 1	50°C	15 min
Step 2	95°C	2 min
45 cycles of Steps 3 and 4		
Step 3	95°C	3 s
Step 4	55°C	30 s ^a

^aSignal acquisition occurs in the FAM (fluorescein) channel during step 4.

D. Purification of RTX

1. Transform a T7-based *E. coli* expression strain, such as BL21(DE3), with a RTX expression plasmid that contains a carboxyl terminal six histidine tag for purification.
2. On the next day, pick an individual transformant colony and inoculate it into desired sterile media (10. Superior BrothTM) with appropriate antibiotics (e.g., Carbenicillin or Ampicillin 100 µg/ml) and 1-2% glucose Grow this starter culture overnight in shaking incubator at 37°C.
3. On day three, inoculate 1 L sterile Superior BrothTM (Athena Enzyme SystemsTM) or other rich media with appropriate antibiotics (e.g., Carbenicillin or Ampicillin 100 µg/ml) in a 4 L Erlenmeyer flask with 250-350 µl of the overnight starter culture. Incubate at 37°C, 220-250 rpm until the culture reaches OD₆₀₀ of 0.4-0.7 (0.5-0.6 is ideal).
4. When the desired OD₆₀₀ is reached, place flask(s) in 4°C cold room for 30-45 min and set shaking incubator to 18°C. Following cold incubation, induce expression of RTX by adding 1 ml of 1 M IPTG to each 1 L of culture. Place flask(s) in the 18°C shaking incubator for 16-18 h.
5. Following overnight expression, harvest cells by centrifuging cultures at 4°C, 5,000 × g for 20 min.
6. On the ice, resuspend cell pellet in 30 ml cold **Lysis Buffer**. Transfer the resuspended cell pellet to a small 50 ml beaker with a clean stir bar and place securely in an ice bath. With moderate stirring, sonicate the sample using 40% amplitude and 1 s ON/4 s OFF for 4 min total sonication time.
7. Centrifuge the resulting lysate at 4°C, 35,000 × g for 30 min. Carefully transfer the supernatant to a clean ultracentrifugation tube, shake in thermomixer at 400 rpm, 85°C for 10 min, and then place on ice for 10 min.
8. Centrifuge the heat-treated lysate at 4°C, 35,000 × g for 30 min. Carefully transfer the supernatant to a clean tube and filter the clarified lysate using a 0.2 µm filter.
9. Prepare Ni-NTA agarose columns with a final column volume (CV) of 1 ml using 10 ml PierceTM disposable columns. Equilibrate column with 20 CV **Equilibration Buffer**.
10. Apply the clarified lysate from 1 L of expressed culture to a previously equilibrated column and collect the flow-through.
11. Wash column with 20 CV **Equilibration Buffer** and collect the flow-through. Wash column with 5 CV **Wash Buffer** and collect flow-through.
12. Elute RTX from a column with 5 ml **Elution Buffer** and transfer to a dialysis cassette with appropriate molecular weight cut-off. Dialyze eluate into 2 L of **Ni-NTA Dialysis Buffer** for 3-4 hours at 4°C. Then dialyze eluate into a second 2 L of **Ni-NTA Dialysis Buffer** overnight at 4°C.
13. Pass the dialyzed eluate over an equilibrated 5 ml heparin column (HiTrapTM Heparin HP) and elute along a sodium chloride linear gradient (100 mM to 2 M NaCl) generated from **Heparin buffer A** and **Heparin buffer B** mixed by an FPLC machine. The peak corresponding to RTX or its variants could be expected between 40-60% Buffer B.
14. Collect fraction tubes containing RTX peaks from the fraction collector. Pool the fractions and dialyze into 2 L **Heparin Dialysis Buffer** for 3-4 h. Then transfer dialysis cassettes into 2 L **Final**

Dialysis Buffer overnight where it is expected that the protein sample volume will decrease significantly. Following the final dialysis, recover the protein sample and determine protein concentration before storing at -20°C. The RTX stock is typically kept concentrated (5-10 mg/ml) in the **Final Dialysis Buffer**. If a lower concentration is required (e.g., 0.2 mg/ml working solution), **Final Dialysis Buffer** was used to dilute the RTX stock to the desired final concentration. An example of RTX purification is shown in **Figure 7**.

15. A similar protocol should work for Taq and other thermostable DNA polymerases, but may require different dialysis and storage buffers.

Data analysis

Analyze RT-qPCR data using LightCycler96 software.

Example data for dye-based RTX-only RT-qPCR

Representative results of SARS-CoV-2 RT-qPCR tests performed using synthetic RNA templates and only RTX or RTX Exo- DNA polymerases are depicted in **Figures 1 and 2**. These results demonstrate that RTX DNA polymerase alone, with or without proofreading capability, can support dye-based RT-qPCR analyses. In our hands, the full-length RTX DNA polymerase was slightly better than the Exo- version, especially in the N3 assay.

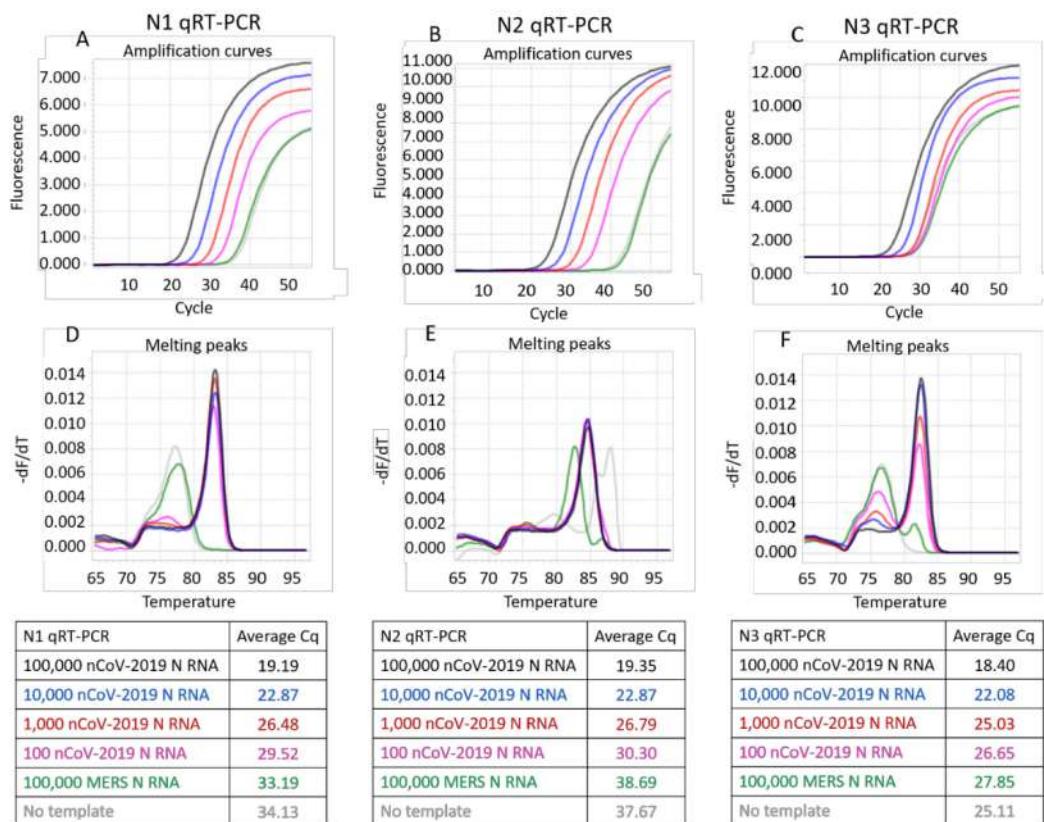


Figure 1. CDC SARS-CoV-2 N1, N2, and N3 RT-qPCR assays performed using indicated

copies of synthetic RNA and RTX DNA polymerase. Panels A, B, and C depict N1, N2, and N3 assays measured in real-time using EvaGreen dye. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Panels D, E, and F depict melting peaks of amplicons determined using the ‘Tm calling’ analysis in the LightCycler96 software. Note that the amplicons observed in negative controls were spurious, as indicated by their distinct melting temperatures compared to target-derived amplicons. Average Cq values of all assays are tabulated.

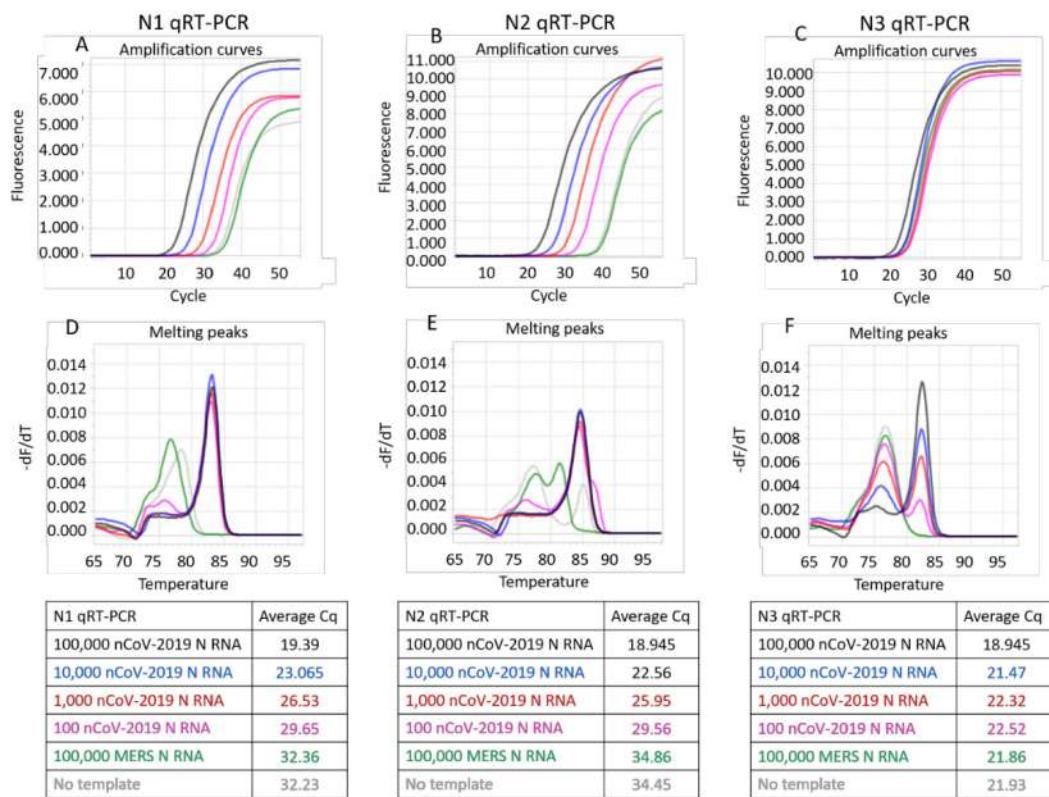


Figure 2. CDC SARS-CoV-2 N1, N2, and N3 RT-qPCR assays performed using indicated copies of synthetic RNA and RTX Exo- DNA polymerase. Panels A, B, and C depict N1, N2, and N3 assays measured in real-time using EvaGreen dye. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Panels D, E, and F depict melting peaks of amplicons determined using the ‘Tm calling’ analysis in the LightCycler96 software. Average Cq values of all assays are tabulated.

Example data for RTX polymerase-based TaqMan RT-qPCR

Representative results of RTX polymerase-based SARS-CoV-2 TaqMan RT-qPCR tests performed using synthetic RNA templates are depicted in **Figures 3 and 4**. Both RTX and RTX Exo- when combined with Taq DNA polymerase are able to support one-pot TaqMan RT-qPCR assays for all three CDC SARS-CoV-2 assays. Under both buffer conditions tested, 1× ThermoPol versus 1× RTX buffer, RTX polymerase yielded more consistent amplification curves for all three CDC assays compared to RTX Exo- polymerase.

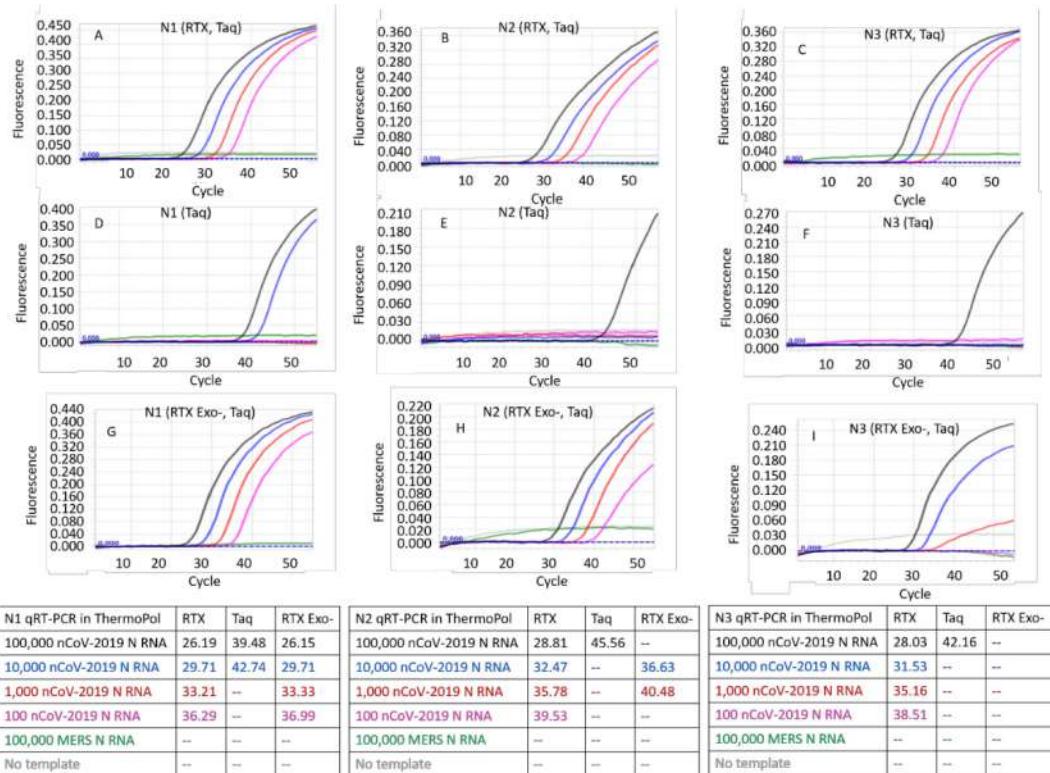


Figure 3. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed in 1X ThermoPol buffer using indicated copies of synthetic RNA and RTX or RTX Exo- and Taq DNA polymerases. Panels A, B, and C depict TaqMan assays containing both RTX and Taq DNA polymerases. Panels D, E, and F depict TaqMan assays containing only Taq DNA polymerase. Panels G, H, and I depict TaqMan assays containing RTX Exo- and Taq DNA polymerases. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

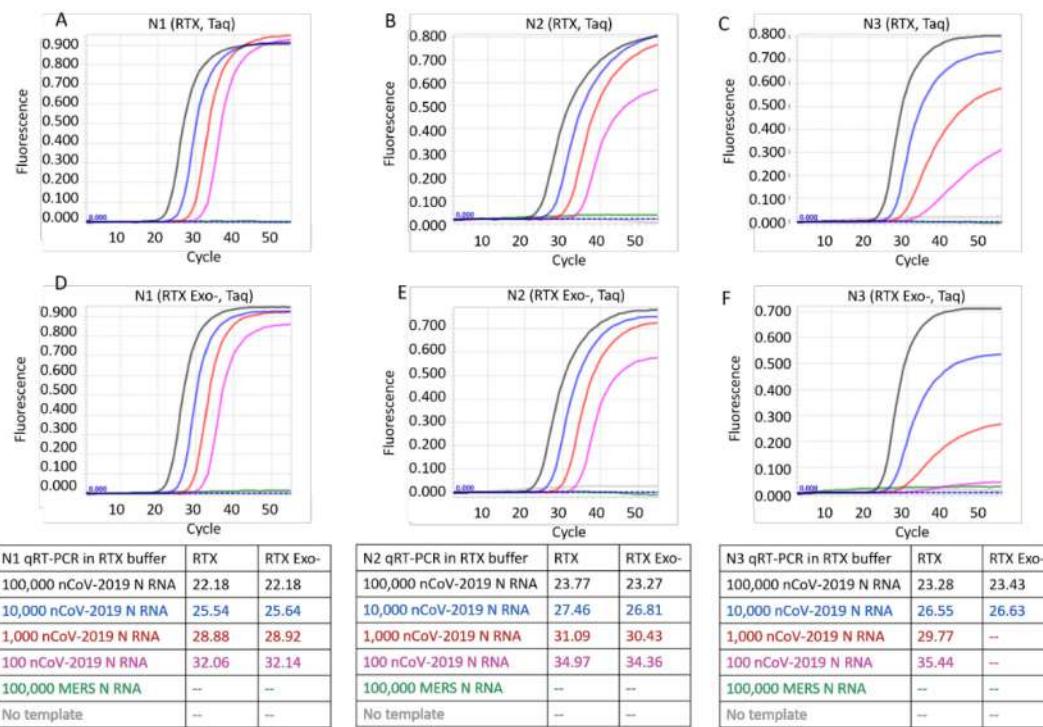


Figure 4. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed in 1X RTX buffer using indicated copies of synthetic RNA and RTX or RTX Exo- and Taq DNA polymerases. Panels A, B, and C depict TaqMan assays containing RTX and Taq DNA polymerases. Panels D, E, and F depict TaqMan assays containing RTX Exo- and Taq DNA polymerases. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

Example data for TaqPath™ TaqMan RT-qPCR

Figure 5 depicts typical results from CDC N1, N2, and N3 TaqMan qRT-PCR reactions performed using synthetic RNA and TaqPath™ commercial RT-qPCR mastermix.

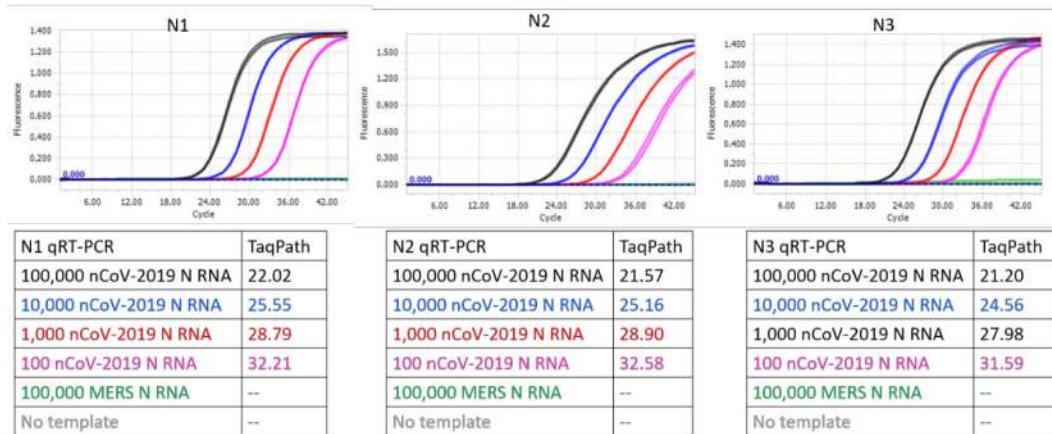


Figure 5. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed using indicated copies of synthetic RNA and TaqPath™ commercial RT-qPCR mastermix. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

The results with either a viral RT (in TaqPath™ commercial mastermix) or RTX are summarized in **Figure 6**. As can be seen, RTX-based TaqMan assays are of comparable sensitivity and specificity to the gold standard TaqPath assay. While all three RTX-based TaqMan assays performed in ThermoPol buffer consistently yielded Cq values, these were somewhat delayed compared to Cq values obtained with the commercial TaqPath™ Master Mix. In contrast, RTX-based TaqMan assays when executed in RTX buffer yielded Cq values that were closer to Cq values obtained with the commercial mastermix.

N1 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	26.19	22.18	22.02
10,000 nCoV-2019 N RNA	29.71	25.54	25.55
1,000 nCoV-2019 N RNA	33.21	28.88	28.79
100 nCoV-2019 N RNA	36.29	32.06	32.21
100,000 MERS N RNA	--	--	--
No template	--	--	--

N2 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	28.81	23.77	21.57
10,000 nCoV-2019 N RNA	32.47	27.46	25.16
1,000 nCoV-2019 N RNA	35.78	31.09	28.90
100 nCoV-2019 N RNA	39.53	34.97	32.58
100,000 MERS N RNA	--	--	--
No template	--	--	--

N3 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	28.03	23.28	21.20
10,000 nCoV-2019 N RNA	31.53	26.55	24.56
1,000 nCoV-2019 N RNA	35.16	29.77	27.98
100 nCoV-2019 N RNA	38.51	35.44	31.59
100,000 MERS N RNA	--	--	--
No template	--	--	--

Figure 6. Comparison of Cq values of RTX-based and TaqPath-based SARS-CoV-2 TaqMan RT-qPCR assays

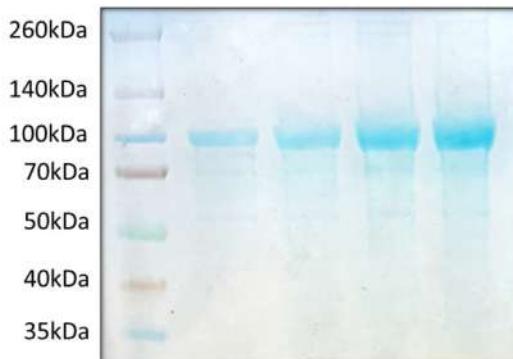


Figure 7. An example of RTX purification analyzed by SDS-PAGE. A total of ten to forty micrograms of purified RTX was developed on a NuPAGE™ 4 to 12%, Bis-Tris mini protein gel. RTX bands appear near the 100kDa size marker. 1st lane: 10 µg, 2nd lane: 20 µg, 3rd lane: 30 µg, 4th lane: 40 µg.

Notes

Overall, RTX performs well either as a single enzyme for RT-qPCR with intercalating dyes, such as EvaGreen, or as the RT component of a TaqMan based assay. At the lowest RNA concentrations examined (100 copies), the gold standard TaqPath assay generally gave a signal at a Cq value of ca. 32. The more robust full-length RTX on its own actually performed slightly better, with Cq values typically from 29-30 (although the N3 primer set gives a higher signal and higher background). When

used as a substitute for a viral RT, RTX is slightly less sensitive overall, with Cq values typically from 32-35.

By obtaining a RTX expression vector and purifying the thermostable reverse transcriptase it should prove possible to carry out RT-qPCR reactions with a sensitivity similar to that already observed for approved kits, with few or no false positive results. Plasmids and sequence information for 6xHis tagged RTX and RTX Exo- protein expression are available from Addgene (pET_RT_X: <https://www.addgene.org/102787/> and pET_RT_X_(exo-): <https://www.addgene.org/102786/>), or can be obtained via <https://reclone.org/>.

Recipes

1. 10× RTX buffer
600 mM Tris-HCl
250 mM (NH₄)₂SO₄
100 mM KCl
20 mM MgSO₄
pH 8.4, 25°C
2. 10× ThermoPol buffer
200 mM Tris-HCl
100 mM (NH₄)₂SO₄
100 mM KCl
20 mM MgSO₄
1% Triton® X-100
pH 8.8, 25°C

Buffers for RTX DNA polymerase purification

3. Resuspension Buffer
50 mM Phosphate Buffer, pH 7.5
300 mM NaCl
20 mM imidazole
0.1% Igepal CO-630 (non-toxic Non-ident P40 equivalent)
5 mM MgSO₄
4. Equilibration Buffer
50 mM Phosphate Buffer, pH 7.5
300 mM NaCl 20 mM imidazole
5. Lysis Buffer
30 ml Resuspension Buffer
1× EDTA-free protease inhibitor tablet
30-60 mg HEW Lysozyme

6. Wash Buffer

50 mM Phosphate Buffer, pH 7.5

300 mM NaCl

50 mM imidazole

7. Elution Buffer

50 mM Phosphate Buffer, pH 7.5

300 mM NaCl

250 mM imidazole

8. Heparin Buffer A

40 mM Tris-HCl, pH 7.5

100 mM NaCl

0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

9. Heparin Buffer B

40 mM Tris-HCl, pH 7.5

2 M NaCl

0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

Buffers for dialysis of RTX DNA polymerase

10. Ni-NTA Dialysis Buffer (2 L ×2)

40 mM Tris-HCl, pH 7.5

100 mM NaCl

10 mM beta-mercapto ethanol (BME) or 1 mM 1,4-Dithiothreitol (DTT)

0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

11. Heparin Dialysis Buffer (2 L)

50 mM Tris-HCl, pH 8.0

50 mM KCl

0.1% Tween-20

0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

12. Final Dialysis Buffer (1 L)

50% Glycerol

50 mM Tris-HCl, pH 8.0

50 mM KCl

0.1% Tween-20

0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

1 mM DTT

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Competing interests

The Board of Regents of The University of Texas has licensed IP covering RTX to Promega Corporation.

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A Protocol for Simple, Rapid, and Direct Detection of SARS-CoV-2 from clinical samples, using Reverse Transcribed Loop-Mediated Isothermal Amplification (RT-LAMP)

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[Abstract] SARS-CoV-2 has quickly spread all around the globe causing illness and wide damages. Most countries were unprepared for such a rapid spread and crisis. This led to various strategies for effective control of the new pandemic. A key aspect in all countries was to effectively test the population for the virus. Most countries chose a lockdown strategy in which many workplaces and activities are completely closed, leading to substantial economy costs. Here, we present a protocol we recently developed that allows rapid and simple detection of SARS-CoV-2 for the large population, eliminating costs and involvement of professional teams and laboratories. This protocol is based on Reverse Transcribed Loop-Mediated Isothermal Amplification (RT-LAMP). We tested this protocol directly on patient samples, both nasal and throat clinical swabs as well as saliva. Notably, this protocol is simple, cheap and can be easily applied to other pathogens as well.

Keywords: SARS-CoV-2, Covid-19, RT-LAMP, Pandemic, Rapid molecular Detection, Colorimetric, Isothermal, Saliva

[Background] The Covid-19 pandemic, caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is affecting large populations, and has been declared a pandemic by the World Health Organization (WHO).

Mass surveillance of the population and quarantine proved to be effective strategies in dealing with this crisis. The main key for detection is the reverse transcription quantitative polymerase chain reaction (RT-qPCR) test. While this test is effective, it requires professional experience both in sampling and in performing the test. Furthermore, the reagents and lab equipment are expensive (Bruce *et al.*, 2020). Altogether, these created a bottleneck for mass scale testing.

Fortunately, to date, alternative molecular biology methods can overcome such limitations. One of these methods is colorimetric Loop-Mediated Isothermal Amplification (LAMP) (Notomi *et al.*, 2000). LAMP is performed at a single and constant temperature (*i.e.*, isothermal), and allows a one-step reverse transcription. Its results can be visualized by color change with the naked eye. This method is cheap and requires little to no lab equipment (Wang *et al.*, 2016; Yu *et al.*, 2020; Zhang *et al.*, 2020). It can be widely used in points of care such as the workplace and schools, which can increase the number of tested subjects tremendously. We see this method as a surveillance tool to markedly increase the total number of tests per day and identify patients for further tests in hospital settings.

Here we present a protocol for applying one step reverse transcribed LAMP (RT-LAMP) detection method on clinical samples from nasal, throat swabs and from saliva. The primers we used were previously designed by Zhang *et al.*, 2020 on synthetic and purified RNA (Table 1). The protocol we present (Ben-Assa*, Naddaf* *et al.*, 2020), while with these same primers, does not require RNA purification steps and can be conducted directly on clinical samples. This protocol requires no professional experience and results can be obtained within an hour. The results of this protocol were compared to the approved RNA purification and quantification RT-qPCR method at the Rambam Health Care Campus (RHCC) hospital. The downside of this method is its detection sensitivity, which is considerably lower than the standard RT-qPCR method (Ben-Assa*, Naddaf* *et al.*, 2020). Therefore, it is applicable for a large-scale surveillance tool rather than a replacement of the current gold-standard detection method. While this protocol was validated on SARS-CoV-2, it can be adjusted to other pathogens too (bacteria or viruses).

Material and Reagents

1. Personal protective equipment (PPE) appropriate for working with SARS-CoV-2
2. 0.2 ml PCR tube strips (Labcon, catalogue number: LC 3927-550)
3. Pipette tips
4. WarmStart® Colorimetric LAMP 2× Master Mix (New England BioLabs Inc., catalog number: M1800) (aliquot and store at -20°C)
5. Proteinase K (Seegene, catalog number: 744300.4.UC384, store at -20°C)
6. Guanidine hydrochloride (Sigma, catalog number: G4505)
7. DNase RNase free water (Biological Industries, catalog number: 01-869-1B)
8. Primers for SARS-CoV-2 (Table 1) (Zhang *et al.*, 2020)
9. Primers for *pop7*-positive internal processing and amplification control (Table 2) (Curtis *et al.*, 2018)

10. Primer mix (see Recipes)
11. Proteinase K (see Recipes)
12. Guanidine hydrochloride (see Recipes)

Table 1. SARS-CoV-2 Primers for RT-LAMP

Primer Name	Sequence
GeneN-A-F3	TGG CTA CTA CCG AAG AGC T
GeneN-A-B3	TGC AGC ATT GTT AGC AGG AT
GeneN-A-LF (Loop Forward)	GGA CTG AGA TCT TTC ATT TTA CCG T
GeneN-A-LB (Loop Backward)	ACT GAG GGA GCC TTG AAT ACA
GeneN-A-FIP (Forward Inner Primer)	TCT GGC CCA GTT CCT AGG TAG TCC AGA CGA ATT CGT GGT GG
GeneN-A-BIP (Backward Inner Primer)	AGA CGG CAT CAT ATG GGT TGC ACG GGT GCC AAT GTG ATC T

Table 2. RNaseP *pop7* Primers for RT-LAMP–Positive Control

Primer Name	Sequence
<i>pop7</i> -F3	TTG ATG AGC TGG AGC CA
<i>pop7</i> -B3	CAC CCT CAA TGC AGA GTC
<i>pop7</i> -LF (Loop Forward)	ATG TGG ATG GCT GAG TTG TT
<i>pop7</i> -LB (Loop Backward)	CAT GCT GAG TAC TGG ACC TC
<i>pop7</i> -FIP (Forward Inner Primer)	GTG TGA CCC TGA AGA CTC GGT TTT AGC CAC TGA CTC GGA TC
<i>pop7</i> -BIP (Backward Inner Primer)	CCT CCG TGA TAT GGC TCT TCG TTT TTT TCT TAC ATG GCT CTG GTC

Equipment

1. 1 µl-10 µl, 10 µl-50 µl, 20 µl-200 µl pipettes
2. PCR Thermocycler as heat source (Biometra, catalog number: T3000)

Procedure

A. Prepare primer mix (see Recipes)

Prepare two mixes: (1) For SARS-CoV-2 test. (2) For *pop7* positive internal processing and amplification control.

B. Sample lysis and preparation (see Figure 1A)

1. For saliva samples: Resuspend in 1 ml of DNase RNase free water (in the same container of the saliva sample). For throat/nasal swab samples: Use the standard universal transfer media (UTM) for this test.
2. Add 5 µl of the sample (from Step B1) to a PCR tube containing 40 µl of DNase RNase free water.
3. Include a non-template control. Add 5 µl DNase RNase free water instead of the sample.
4. Add 2 µl proteinase K (final concentration 1.22 mg/ml).
5. Incubate at room temperature for 15 min.
6. Incubate samples at 95°C for 5 min in a PCR machine or any other heat source.
Note: We have successfully used thermal cup and hot water.
7. Cool your sample down to room temperature.

C. RT-LAMP reaction (see Figure 1A)

Prepare three mixes: (1) For SARS-CoV-2 test. (2) For *pop7* gene as positive control. For each mix use the relevant primers. (3) For non-template control (see Step B3) using the SARS-CoV-2 primers.

1. Prepare reaction:

LAMP mix	10 µl
Primer mix	2 µl
Guanidine hydrochloride	1 µl
Sample lysate (product of Procedure B)	7 µl

2. Incubate at 65°C for 30-40 min.

3. Read results:

If the mix color changes from pink to yellow, the sample has tested positive (see Figure 1B).

If the mix maintains its pink color, the sample has tested negative (see Figure 1B).

Please note that the results are binary as positive or negative. Only bright yellow results are considered positive. Pink is considered negative, and any other range or gradient of color is considered negative.

Positive control results were published at Ben-Assa*, Naddaf* et al., 2020 (Figure 3a).

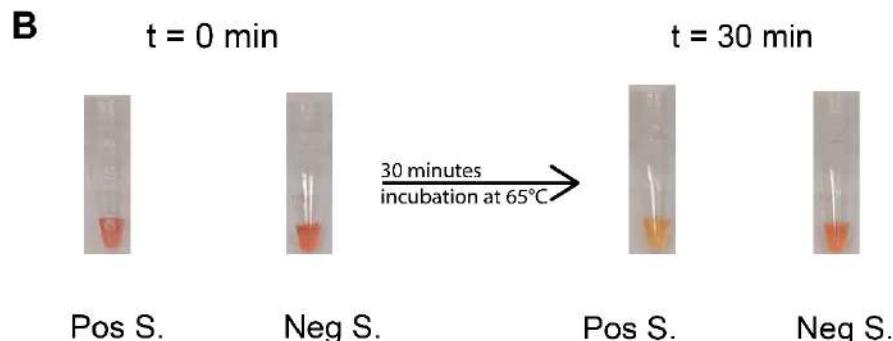
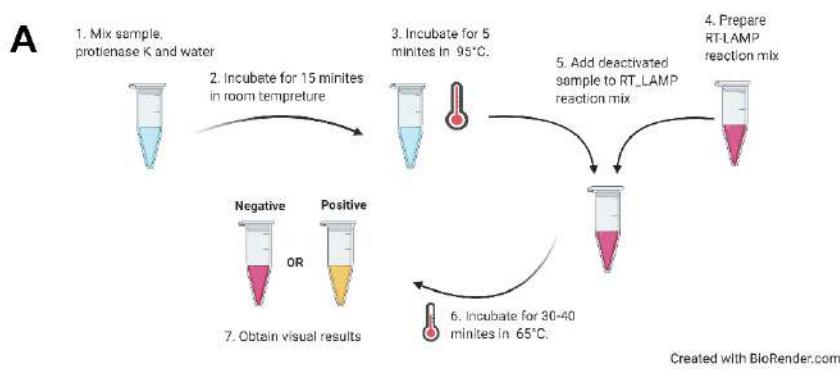


Figure 1. Procedure and results of SARS-CoV-2 RT-LAMP detection test. A. An illustration of the steps of the protocol: this illustration summarizes the main steps of the protocol. Illustration was created with BioRender.com. B. One negative sample (Neg S.) and one positive sample (Pos S.) before and after incubation. At $t = 0 \text{ min}$ (left panel), all samples were pink. After incubation of 30min (right panel), positive samples turned yellow while negative samples retained their pink color. Pictures are representative RT-LAMP test results of clinical diagnostic nasal and throat swabs of data published in Ben-Assa*, Naddaf* et al., 2020. Results were confirmed by conventional RT-qPCR clinical test for SARS-CoV-2 following RNA extraction and purification step.

Notes

1. Alternative to a PCR machine, any heating source (e.g., water bath) can be used for Steps B5 and C2. We have successfully used a thermos cup and a thermometer to adjust water temperature.
2. Longer incubation than 40 min may result in non-specific color change and interpreted as false positive results.
3. It is very important to use proper personal protective equipment when handling the samples starting from step B and throughout the rest of the protocol.
4. The color change in the protocol is an indication for a binary positive/negative result and does not represent any gradient indication of the clinical status of the patient.

Recipes

1. Primer mix
 - a. Prepare each one of the primers at 100 µM
 - b. Primer mix:

F3	2 µl (2 µM final concentration)
B3	2 µl (2 µM final concentration)
LF	4 µl (4 µM final concentration)
LB	4 µl (4 µM final concentration)
FIP	16 µl (16 µM final concentration)
BIP	16 µl (16 µM final concentration)
 - c. DNase RNase free water 56 µl
 - d. Aliquot and store at -20°C
2. Proteinase K at stock concentration 28.67 (mg/ml)
Soluble in DNase RNase free water
Store at -20°C
3. Guanidine hydrochloride
Soluble in DNase RNase free water
Stock concentration, 800 mM
Store at room temperature

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Competing interests

The author(s) declared 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 was granted exemption from IRB approval of the Rambam Health Care Campus (# 0244-20-RMB) for use of de-identified COVID-19 tests performed for the purpose of the standard testing, and for 4 volunteers.

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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

[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 μ l each of the 10 clinical NP/OP swabs into a single 1.5-ml microcentrifuge tube. The total volume is 600 μ l. The original volume of the specimens ranged from 1 ml to 3 ml.
3. Aliquot 300 μ l pooled swabs to each of two clean 1.5-ml microcentrifuge tubes and label with the same sample ID.

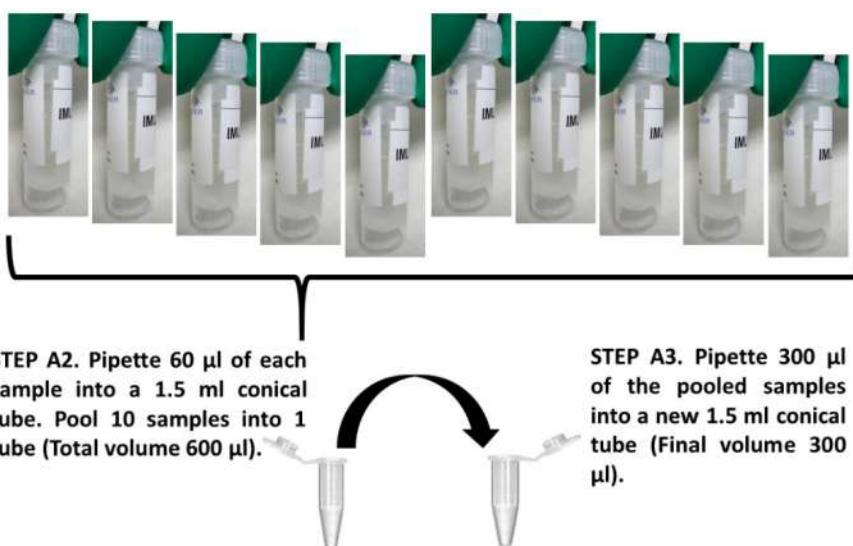


Figure 1. Pooling of 10 individual samples

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 μ l 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 μ l 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 μ l lysate mixture to the VB column in a 2-ml collection tube. Centrifuge at

- 14,000 × 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 × 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 × g for 30 s. Discard the flowthrough and place the VB column back into the 2-ml collection tube.
 - vii. Centrifuge at 14,000 × 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 (15–25°C).
 - ix. Centrifuge at ≥6,000 × 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 ≥6,000 × 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 ≥6,000 × 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 × 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 ≥6,000 × 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 (μ l) for one reaction
2× reaction buffer	1×	10.0
SuperScript™ III RT/Platinum™ Taq Mix	-	0.5
RdRP-Primer/Probe mixture (10 μ M)	2 μ M	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 ≤ 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 ≤ 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 μ l) and 6 (total volume 360 μ l), 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 29th, 2020. IMU COVID-19 pooled testing results

No.	Group	Pooled samples (10 specimens), IMU Ref#	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	COVID19 Detected (23.71) (9 samples + internal +ve control)	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 μ l) and 6 (total volume 360 μ l), respectively. Aliquots of 150 μ l (Group J) and 180 μ l (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		Not Detected
5	1462	COVID19 Detected (23.71)	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
 - a. Prepare each one of the primers and probes at 100 μM
 - b. 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)
 - c. DNase- and RNase-free water 60 μl
 - d. Aliquot and store at -20°C

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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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Detection of the SARS-CoV-2 Nucleocapsid Protein (NP) Using Immunohistochemistry

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[Abstract] While lymphocytopenia is a common characteristic of patients infected by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the mechanisms responsible for this depletion are unclear. With the tissue samples of the spleens and lymph nodes (LNs) from six cases, immunohistochemistry demonstrated ACE2 (angiotensin-converting enzyme 2), the potential receptor of SARS-CoV-2, expresses on tissue-resident CD169⁺ macrophages in spleens and LNs.

Keywords: SARS-CoV-2, ACE2, Spleen, Lymph nodes, Immunohistochemistry

[Background] In December 2019, clusters of patients with pneumonia of unknown etiology were reported by the local health facilities in Wuhan, Hubei Province, China (Li *et al.*, 2020; Zhu *et al.*, 2020). In January 2020, the causative agent of mysterious pneumonia was identified as a novel coronavirus named as 2019 novel coronavirus (2019-nCoV) by the World Health Organization (WHO). This novel coronavirus was renamed as the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and the consequent disease was designated as coronavirus disease 2019 (COVID-19) (Lu *et al.*, 2020; Xu *et al.*, 2020). According to the daily report of the WHO, the epidemic of SARS-CoV-2 has so far caused 81961 laboratory confirmed cases and 3293 death in China. Meanwhile, the number of confirmed COVID-19 and fatalities around the world are 465315 and 21031, respectively, by 26th March 2020 (World Health Organization, 2020).

The importance of the secondary lymphoid organs, including spleen and lymph nodes (LNs), for resistance against infection is well established. Tissue-resident macrophages positioned in the splenic marginal zone (MZ) that are among the first cell types to encounter invading pathogens (Aichele *et al.*, 2003; Mebius and Kraal, 2005). Similarly, the resident macrophages of the subcapsular sinus and hilar lymph nodes have been shown to play a protective role against viral infections by capturing viral particles (Junt *et al.*, 2007; Tamburini *et al.*, 2014). These macrophages present viral antigens to activate specific T cells, to induce activation, clonal proliferation, and

subsequent killing of infected target cells through secretion of perforin, granzyme B, and interferon- γ etc (Backer *et al.*, 2010; Bernhard *et al.*, 2015; van Dinther *et al.*, 2018). Moreover, these activated T cells also egress from spleen and LNs into blood circulation and play immune surveillance roles (Gebhardt *et al.*, 2013). Unfortunately, our previous work has shown that lymphocytopenia is prevalent in COVID-19 patients, especially in aged and critically ill cases, suggesting that the immune system from COVID-19 patients might be neutralized by infection (Diao *et al.*, 2020).

Therefore, understanding the destinies of lymphocytes in secondary lymphoid organs is critical for understanding SARS-CoV-2 viral infection, and the identification of methods to boost lymphocytes and enhance the host immunity *in vivo*.

In this study, the tissue samples of spleens and LNs are from six COVID-19 patients. Viral nucleocapsid protein (NP) antigen, cell apoptosis and proinflammatory cytokine expression were measured by immunohistochemistry. And the protocol is detailed below.

Materials and Reagents

1. Glass slides
2. Paraffin-embedded tissue blocks
3. Poly-L-lysine
4. Hydrogen peroxidase
5. Methanol
6. BSA
7. PBS
8. Nonidet P-40
9. Hematoxylin
10. Ethanol
11. The Vecta-stain ABC kit (Vector Laboratories, San Diego, CA, USA)
12. DAB Elite kit (DAKO, Copenhagen, Denmark, catalog number: K3465)
13. Antibodies
 - Anti-SARS-CoV-2 nucleocapsid protein (NP) antibodies (1:100, rabbit IgG) (Sino Biological, Beijing, clone ID: 019)
 - Anti-ACE2 (1:100, rabbit IgG) (Sino Biological, clone ID: 10108-RP01)
 - Anti-CD68 (1:100, mouse IgG1) (BIO-RAD, Clone ID:KP1)
 - Anti-CD169 (1:100, mouse IgG1) (Biolegend, Clone ID:7-239)
 - Anti-B220 (1:100, mouse IgG1) (BIO-RAD, Clone ID:123C3)
 - Anti-Fas (1:100, mouse IgG) (Thermo fisher, ID:48095942)
 - Anti-FasL (1:100, rabbit IgG) (Santa Cruz, catalog number: sc-834)
 - Anti-IL-6 (1:100, mouse IgG2b) (Santa Cruz, catalog number: sc-130326)
 - Rabbit-isotype antibody controls (1:100) (Dako)
14. 10 mM citrate buffer (pH = 6.0) (see Recipes)
15. 0.5% hydrogen peroxidase (H₂O₂) (see Recipes)

Equipment

1. Light microscope (Zeiss Axioplan 2)

Procedure

In the protocol, tissue samples of the spleens and LNs from six COVID-19 patients were collected and used upon the approval of the National Health Commission of China and Ethics Commission of General Hospital of Central Theatre Command and Jinyintan Hospital.

1. Cut the paraffin-embedded tissue blocks into 2-3 μm sections.
2. Mount them on poly-L-lysine-charged glass slides.
3. De-wax and rehydrate the sections
4. Add the sections in 10 mM citrate buffer (pH 6.0) and perform the antigen retrieval with microwave.
5. Incubate with a solution of 0.5% H₂O₂ in 50% methanol for 1 h to block the endogenous peroxidase activity.
6. Incubate in 3% BSA plus 0.1% Nonidet P-40 in PBS for 1 h at room temperature. So that the nonspecific binding is blocked.
7. Dilute the primary antibodies with 1% BSA.

Notes: The primary antibodies consist of anti-SARS-CoV-2 nucleocapsid protein (NP) antibodies, anti-ACE2, anti-CD68, anti-CD169, anti-B220, anti-Fas, anti-FasL, anti-IL-6 or rabbit-isotype antibody controls (1:100; Dako).

8. Incubate the sections in the diluted primary antibodies overnight at 4°C.
9. Wash the sections.
10. Incubate the sections with the corresponding secondary antibodies for 1 h at room temperature.
11. Perform the avidin- biotin complex method with the Vecta-stain ABC kit. Detailed procedures are followed with the manufacturer's instructions.
12. Incubate the sections with isotype-matched concentration-matched immunoglobulin.

Note: No primary antibodies are used as isotype controls.

13. Visualize the peroxidase activity with the DAB Elite kit.

Note: Brown coloration of tissues represents positive staining.

14. Counterstain the sections with hematoxylin.
15. Dehydrate the sections through an ethanol series to xylene and mount them.
16. View the sample sections with a light microscope (Figure 1).

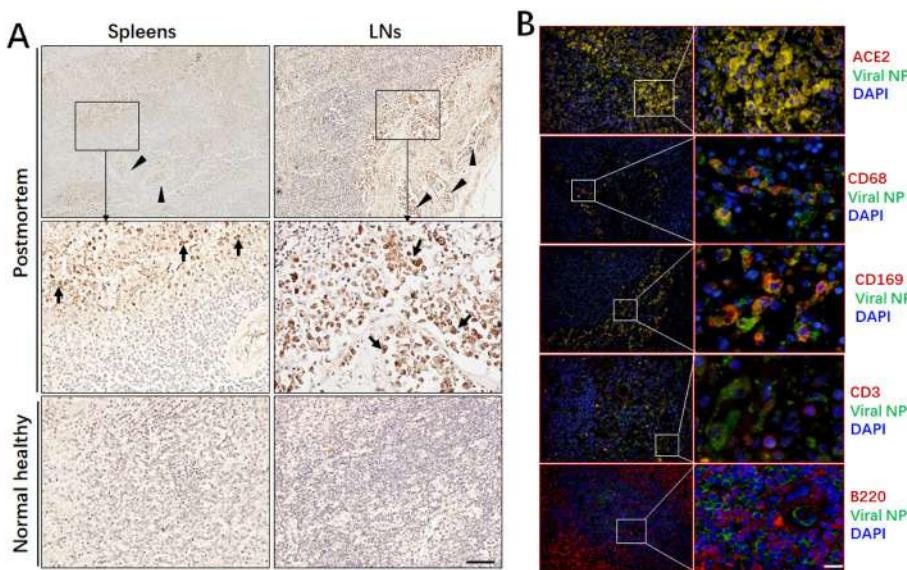


Figure 1. Immunohistochemistry analyzed SARS-CoV-2 NP antigen in spleen and LN tissues. A. The expression of viral NP antigen was detected by immunohistochemistry in spleen and LN tissues from COVID-19 patients undergoing postmortem examination and age- match normal healthy controls. B. Immunofluorescent double staining analyzed viral NP antigen expression. Arrow indicated viral NP positive cells. Scale bar= 100 μ m. (Chen et al., 2020)

Recipes

1. 10 mM citrate buffer (pH = 6.0)
2. 0.5% hydrogen peroxidase (H₂O₂)

Acknowledgments

Yuzhang Wu, and Yongwen Chen were involved in the final development of the project and manuscript preparation; Zilin Yuan, Chenghui Wang and Zeqing Feng analyzed the data; Bo Diao, Yin Liu, Gang Wang, Yinjun Tan and Yueping Liu did H&E staining and immunohistochemistry; Changsong Wang evaluated H&E and immunohistochemistry results; Liang Liu, Rongshuai Wang and Liang Ren provided autopsies and analyzed H&E staining.

Competing interests

The authors declare no financial or commercial conflict of interest.

Ethics

This study was approved by the National Health Commission of China and Ethics Commission of General Hospital of Central Theatre Command and Jinyintan Hospital.

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Colorimetric RT-LAMP and LAMP-sequencing for Detecting SARS-CoV-2 RNA in Clinical Samples

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[Abstract] During pandemics, such as the one caused by SARS-CoV-2 coronavirus, simple methods to rapidly test large numbers of people are needed. As a faster and less resource-demanding alternative to detect viral RNA by conventional qPCR, we used reverse transcription loop-mediated isothermal amplification (RT-LAMP). We previously established colorimetric RT-LAMP assays on both purified and unpurified SARS-CoV-2 clinical specimens and further developed a multiplexed sequencing protocol (LAMP-sequencing) to analyze the outcome of many RT-LAMP reactions at the same time (Dao Thi et al., 2020). Extending on this work, we hereby provide step-by-step protocols for both RT-LAMP assays and read-outs.

Keywords: RT-LAMP, LAMP-sequencing, SARS-CoV-2 detection, Tn5 tagmentation, colorimetric assay

[Background] The new SARS-CoV-2 coronavirus poses a major public health problem (reviewed in Li et al., 2020). In the absence of efficient antiviral treatments and a protective vaccine, preventing local outbreaks by mass testing is critical. The standard diagnostic pipeline to detect SARS-CoV-2 infections is based on the isolation of viral RNA from clinical specimens, a reverse-transcription (RT) reaction to transcribe the RNA into cDNA, and detection by a semi-quantitative DNA polymerase chain reaction (qPCR) (Corman et al., 2020). Yet, commercial RNA isolation and RT-qPCR kits are costly, time-consuming, and shortages of supplies during the pandemics limit high-throughput testing requiring alternative solutions (Klein et al., 2020).

In our recent study (Dao Thi et al., 2020), we used reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Notomi et al., 2020) as an alternative to detect SARS-CoV-2 RNA in clinical specimens. We developed and characterized colorimetric RT-LAMP assays on both purified and unpurified pharyngeal swab specimens. We also developed a multiplexed sequencing protocol based on tagmentation for enzymatic addition of barcoded sequencing library adapters. This enables the analysis of many RT-LAMP reactions at the same time. Here, we present detailed step-by-step protocols to further facilitate the application of RT-LAMP for mass testing.

Materials and Reagents

1. 1.5 ml tubes (Eppendorf), room temperature
2. Filter tips (for pipettes and liquidator), room temperature
3. 96-well plate (Eppendorf, catalog number: 0030128672), room temperature
4. Nuclease-free water (Ambion, catalog number: AM9937), room temperature
5. Ethanol for Molecular Biology
6. WarmStart Colorimetric RT-LAMP 2× Master Mix (New England Biolabs, catalog number: M1800), -20°C
7. 10× primer mix for RT-LAMP assay as in Table 1 (Sigma-Aldrich), -20°C

Table 1. N gene primer for RT-LAMP assay. Primer sequences were designed by Zhang *et al.* (2020).

Name	Sequence	Concentration in 10× primer mix (μM)
GeneN-A-F3	TGGCTACTACCGAAGAGCT	2
GeneN-A-B3	TGCAGCATTGTTAGCAGGAT	2
GeneN-A-FIP	TCTGGCCCAGTTCCTAGGTAGTCCAG ACGAATTCTGTGGTGG	16
GeneN-A-BIP	AGACGGCATCATATGGGTTGCACGGG TGCCAATGTGATCT	16
GeneN-A-LF	GGACTGAGATCTTCATTTACCGT	4
GeneN-A-LB	ACTGAGGGAGCCTTGAATACA	4

8. LAMP-sequencing primers as in Table 2 (Sigma-Aldrich), -20°C

Table 2. LAMP-sequencing primers. The full table is available as Table S4 in Dao Thi *et al.* (2020). [Phos] = phosphorylation, [SpcC3] = C3 spacer group, N, X, Y indicate one of the bases [GATC] (N are random bases while X and Y belong to respective inline barcodes used for multiplexing).

Name	Sequence
Tn5hY-Rd2-Wat-SC3	[Phos]CTGTCTCTTATACACATCT[SpcC3]
P5-UMI-xi5XXX-ME.fw	CGGCGACCACCGAGATCTACACNNNNNNNNXXXXXXXXXXXXCGT CGGCAGCGTCAGATGTGTATAAGAGACAG
P5.fw	AATGATAACGGCGACCACCGAGATC
P7nxt-GeneN-A-LBrc	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGTATTCAAGGCT CCCTCAGT
P7-xi7YYY	CAAGCAGAAGACGGCATACGAGATYYYYYYYYYYTCTCGTGGGCT CGGAG

9. Optically clear adhesive seal (Kisker Biotech, catalog number: GK480-OS), room temperature

10. Adhesive aluminum foil seal (Steinbrenner Laborsysteme, catalog number: SL-AM0550), room temperature
11. Pierceable foil (Brooks Life Sciences, catalog number: 4ti-0566/96), room temperature
12. 200 ng/ μ l Tn5 (E54K, L372P) Transposase (purified according to Hennig *et al.*, 2018, -80°C)
13. 0.2% SDS solution (room temperature)
14. AMPureXP bead (Beckman Coulter, catalog number: A63881), 4°C
15. NEBNext Q5 HotStart polymerase (New England Biolabs, catalog number: M0543), -20°C
16. NucleoSpin Gel and PCR Clean-up mini kit (Macherey-Nagel, catalog number: 740609), room temperature
17. NEBNext Library Quant Kit for Illumina (New England Biolabs, catalog number: E7630), -20°C
18. [Tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS)
19. MgCl₂
20. Dimethylformamide (DMF)
21. Freshly prepared 5 \times fragmentation buffer (see Recipes)

Note: All chemicals purchased from Sigma-Aldrich except when indicated otherwise.

Equipment

1. Pipetman L P2L, 0.2-2 μ l (Gilead, catalog number: FA10001M)
2. Pipetman L P20L, 2-20 μ l (Gilead, catalog number: FA10003M)
3. Pipetman L P200L, 20-200 μ l (Gilead, catalog number: FA10005M)
4. Pipetman L P1000L, 100-1, 000 μ l (Gilead, catalog number: FA10006M)
5. Pipetman L Multichannel P8 x 20L, 2-20 μ l (Gilead, catalog number: FA10009)
6. Liquidator 96 2-20 μ l (Mettler Toledo, catalog number: LIQ-96-20)
7. Thermocycler (Biometra, TAdvanced 96 S)
8. Absorbance reader (Tecan, model: Infinite M200/Spark Cyto)
9. Centrifuge (Eppendorf, model: 5430 R)
10. Table top centrifuge (Heraeus, model: Pico 21)
11. Magnetic stand (6 Tube Magnetic Stand; Ambion, catalog number: 10055)
12. NextSeq 550 machine (Illumina)

Procedure

A schematic diagram depicting the whole experimental procedure is shown in **Figure 1**.

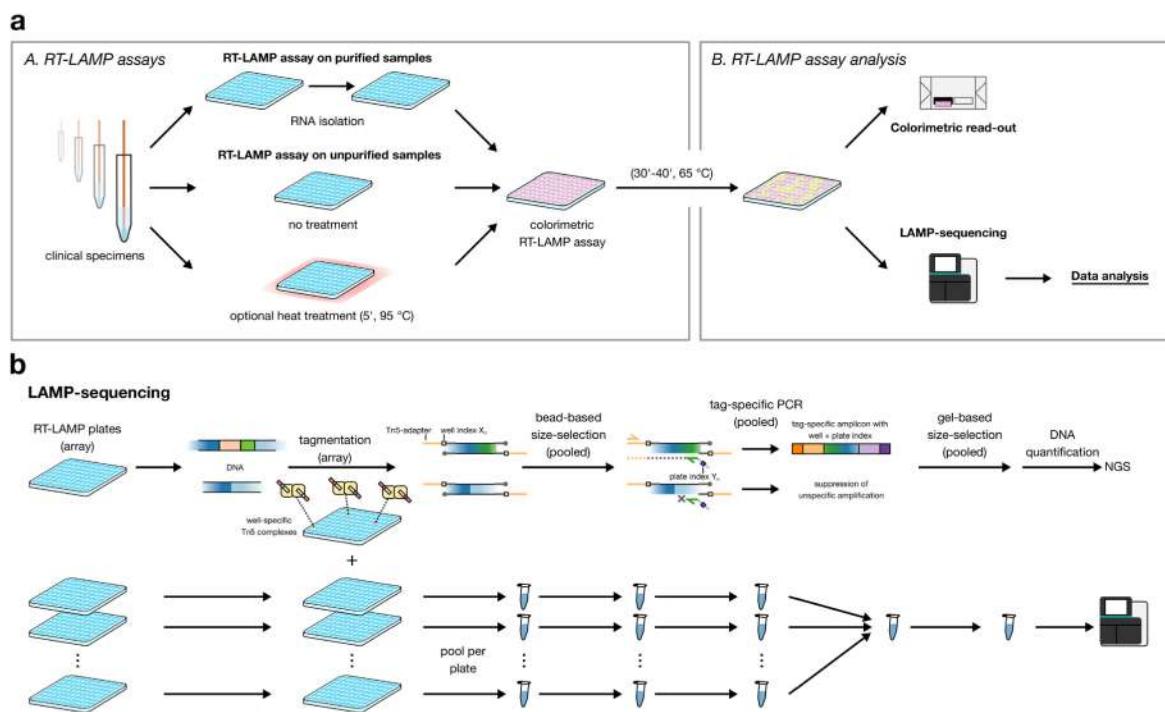


Figure 1. Overview of experimental procedures. (a) RT-LAMP assays can be performed with purified or unpurified clinical specimens and then analyzed using a colorimetric or LAMP-sequencing read-out. **(b)** Flow-chart of LAMP-sequencing library preparation.

A. RT-LAMP assays

RT-LAMP assay on purified samples

1. Isolate RNA from clinical specimen according to manufacturer's protocol.
2. Assemble RT-LAMP master mix in a 1.5 ml Eppendorf tube by adding 6.25 µl of the 2× Master Mix, 1.25 µl of 10× primer mix, and 4 µl nuclease-free water per reaction.
3. Vortex and spin down.
4. Distribute 11.5 µl of master mix into each well of a 96-well plate using a multichannel pipette.
5. Add 1 µl of isolated RNA into wells with master mix.
6. Seal plate with optically clear adhesive seal.
7. Briefly spin down plate.
8. Incubate for 30 min at 65°C in a thermocycler (with the lid heated to 75°C).

RT-LAMP assay on unpurified samples

1. For hot swab-to-RT-LAMP assays, pipette 50 µl of clinical specimen into 96-well plate and seal with pierceable foil.
2. Heat up plate for 5 min at 95 °C in 96-well plate in a PCR cycler (with the lid heated to 105°C).
3. Cool down, spin briefly, and keep plate on ice.
4. Assemble RT-LAMP master mix in a 1.5 ml Eppendorf tube by adding 10 µl of the 2× Master Mix, 2 µl of 10× primer mix, and 7 µl nuclease-free water per reaction.

5. Vortex and spin down.
6. Distribute 19 µl of master mix into each well of a 96-well plate using a multichannel pipette.
7. For direct swab-to-RT-LAMP assays, pipette 1 µl of clinical specimen directly into wells with master mix.
8. For hot assays, pipette 1 µl of prepared specimen (1-3) into wells with master mix.
9. Seal plate with optically clear adhesive seal.
10. Briefly spin down plate.
11. Incubate for 30 min at 65 °C in a thermocycler (with the lid heated to 75°C).

B. RT-LAMP assay analysis

Colorimetric read-out

1. Cool down 96-well plate to 4°C and spin down briefly.
2. Place 96-well plate into absorbance reader.
3. Measure absorbance at 434 nm and 560 nm.

LAMP-sequencing

(All concentrations are given as final concentrations in reactions.)

1. Prepare transposon adapters by mixing individual barcoded adapter (P5-UMI-xi5XXX-ME.fw) with the primer Tn5hY-Rd2-Wat-SC3 at a final concentration of 25 µM per primer in 5 µM Tris-HCl (pH 8.0) in a 96-well PCR plate using the Liquidator. Heat up to 99°C for 5 min and let the primers slowly anneal by cooling down to 20°C within 15 min using a thermocycler.
2. Mix transposase to a final concentration of 100 ng/µl with 1.25 µM annealed adapters from step 1 in 50 mM Tris-HCl (pH 7.5) in 96-well PCR plates using the Liquidator. Assemble transposons by incubating the reaction for 1 h at 23°C in a thermocycler.
3. Freshly prepare the 5× tagmentation buffer according to the indicated composition.
4. Per well mix 1.2 µl of the RT-LAMP product (equivalent to ~200 ng DNA) with 1.5 µl of loaded transposase, 1.12 µl 5× tagmentation buffer from step 3 and 1.8 µl water to assemble the transposon reactions in 96-well PCR plates with the Liquidator. Incubate reactions at 55°C for 10 min in a thermocycler.
5. Stop the tagmentation reactions by adding 1.13 µl 0.2% SDS per well and incubate for 10 min at room temperature. Pool the reactions into one single reaction each plate.
6. Perform size selection for fragments of approximately 300 to 600 bp by using the following two-step AMPure XP bead protocol (written for a pooled reaction from one plate).
 - a. Mix 50 µl of pooled reaction with 50 µl of water.
 - b. Remove large fragments by adding 55 µl of AMPure XP beads to the diluted samples. Mix by pipetting ten times and incubate at room temperature for 5 min. Separate beads from supernatant by placing on a magnetic stand for ~5 min. Transfer the supernatant to a fresh eppendorf tube using a pipette without transferring beads.
 - c. Remove small fragments by adding 25 µl of fresh beads to the supernatant. Mix by pipetting

ten times and incubate at room temperature for 5 min. Separate beads from supernatant by placing on a magnetic stand for ~5 min. Discard the supernatant containing the small fragments using a pipette without disturbing the bead pellet.

- d. Wash DNA bound to beads by two washes with ethanol. For this, add 200 μ l ethanol (80%) to the beads, mix by pipetting ten times and incubate at room temperature for 5 min. Separate beads from ethanol by placing on a magnetic stand for ~5 min. Repeat this for a second wash. Let the beads air-dry for 10 min.
- e. Elute DNA from beads by adding 10 μ l of 5 mM Tris-HCl (pH 8.5), incubating for 5 min at room temperature and separating on a magnetic rack for ~5 min.
7. Perform one PCR reaction per plate using 1 μ l of size-selected eluate from step 6 as a template. Prepare PCR reactions with RT-LAMP-specific and Tn5-adapter-specific primers (P7nxt-GeneN-A-LBrc and P7-xi7YYY, P5.fw) with the NEBNext Q5 HotStart polymerase according to the manufacturer's instruction. Use the following PCR conditions for amplification with a thermocycler: Two cycles at 62°C for annealing and 90 s elongation, followed by two cycles at 65 °C for annealing and 90 s elongation, and 13 cycles at 72°C annealing and 90 s elongation.
8. Pool all PCR reactions and perform a second size selection for fragments of approximately 400 to 550 bp: Run 20% of the pooled PCR reactions on a 2% agarose/Tris-acetate-EDTA gel, cut out the respective part of the lane and use a gel purification kit according to the manufacturer.
9. Quantify the library using for example a qPCR-based library quantification kit.
10. Perform a custom Illumina sequencing run on a NextSeq 550 machine based on the instructions of the manufacturer using 20% phiX spike-in and 136 cycles for the first read, 11 cycles to read the 11-nt-long plate index (i7) and 20 cycles to read the 11-nt-long well index (i5) and the 9-nt-long UMI.

Data analysis

A. Colorimetric RT-LAMP analysis

The results of the colorimetric RT-LAMP assay can be judged by naked eye. A clear color change from pink to orange or yellow is considered as SARS-CoV-2 positive after 30 min incubation at 65°C. Color changes after 30 min can be caused by spurious amplification products and are therefore scored negative. For further validation, the RT-LAMP product can be analyzed by gel electrophoresis and should yield a distinct banding pattern as described previously (Dao Thi et al., 2020, see Figure 1 herein).

When the assay is analyzed by a plate reader, subtract absorbance reads 560 nm from 434 nm (Δ OD). An Δ OD value > 0.3 is considered SARS-CoV-2 positive after 30 min incubation at 65°C. For the hot swab-to-RT-LAMP assays, this read-out can be improved by subtracting the differences between the Δ OD values at time points 30 min and 10 min of the incubation at 65°C.

B. LAMP-sequencing analysis

Raw NGS results (single-end fastq file) need first to be converted to count tables using a workflow, which can be downloaded from GitHub (https://github.com/anders-biostat/LAMP-Paper-Figures/tree/master/LAMP-sequencing_raw_read_processing). All the necessary software to run this workflow are summarized there. Individual processing steps can be run sequentially from inside this directory with the script '00-run_workflow.sh' used for illustration the example file 'LAMP-sequencing_raw_sample100k.fastq.gz'. In order to run the workflow with a different dataset one needs to adapt the pathnames in '00-run_workflow.sh' accordingly. Two files are the result ('counts.tsv' and 'counts.Rda') which can be used for subsequent analysis. For example the count table to produce the respective figures for our RT-LAMP study (Dao Thi *et al.*, 2020) is also present in this GitHub repository.

Notes

All work with crude SARS-CoV-2 clinical specimens should be carried out in a biosafety level 2 cabinet until inactivation. We found that both purified and unpurified pharyngeal swab specimens as well as saliva specimens were compatible with RT-LAMP assays. Other types of specimens have to be tested.

In order to avoid contaminations and RNA degradation, all steps are carried out using filter tips and wearing gloves. In addition, keep clinical specimens on ice as much as possible to prevent RNA degradation. Master mix and test samples should be pipetted at different workplaces using different sets of pipettes. Ideally, the person executing the protocols has experience in molecular biology. Additional important considerations when using RT-LAMP reagents are listed in the Supplementary Material of our previously published work (Dao Thi *et al.*, 2020).

Recipes

1. 5× fragmentation buffer

(Always prepare fresh.)

1 vol of 10× TAPS buffer (100 mM [Tris(hydroxymethyl)methylamino]propanesulfonic acid

(TAPS) (pH 8.5), 50 mM MgCl₂)

1 vol of 100% (v/v) dimethylformamide (DMF)

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Colorimetric RT-LAMP Methods to Detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

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[Abstract] Standard diagnostic methods of Coronavirus Disease 2019 (COVID-19) rely on RT-qPCR technique which have limited point-of-care test (POCT) potential due to necessity of dedicated equipment and specialized personnel. LAMP, an isothermal nucleic acid amplification test (NAAT), is a promising technique that may substitute RT-qPCR for POCT of genomic materials. Here, we provide a protocol to perform reverse transcription LAMP targeting SARS-CoV-2. We adopted both real-time fluorescence detection and end-point colorimetric detection approaches. Our protocol would be useful for screening diagnosis of COVID-19 and be a baseline for development of improved POCT NAAT.

Keywords: SARS-CoV-2, COVID-19, LAMP, RT-LAMP, Colorimetric detection

[Background] Fast and sensitive detection of SARS-CoV-2, the etiologic agent of COVID-19, is important to control current pandemic situation as it enables early detection, isolation and treatment as well as monitoring screening. Current standard methods for detection of SARS-CoV-2 utilize RT-qPCR as World Health Organization recommends (WHO, 2020) However, proper performance of RT-qPCR diagnosis requires high profile facilities and specialists, often not available in the hospital/sampling places, thus, lacking POCT suitability.

Isothermal amplification methods are developed to accomplish diagnosis by nucleic acid detection in various point-of-care as it can be performed with relatively simple instruments (Niemz et al., 2011). Loop-mediated isothermal amplification (LAMP) is one of such isothermal NAAT (Notomi et al., 2000). Amplification by LAMP reaction can be observed through fluorescent dye with real-time PCR instruments (Oscorbin et al., 2016), or through cost effective colorimetric detection methods (Goto et al., 2009; Miyamoto et al., 2015; Tanner et al., 2015). Indeed reverse transcription LAMP (RT-LAMP) may be considered a promising tool as several other groups are employing this technique for SARS-CoV-2 detection (Yan et al., 2020; Zhang et al., 2020).

Here, we present our protocol used to develop RT-LAMP assays targeting SARS-CoV-2. Candidate primer sets are designed using web based tool PrimerExplorerV5 (<https://primerexplorer.jp/e/>) targeting SARS-CoV-2 specific regions compare to SARS-CoV and SARS-CoV-2 Nucleocapsid. The primer sets are

screened for limit of detection and threshold time measured through real time fluorescent method. Below illustrated protocol includes RNA standard preparation, titration of cultured viral RNA and LAMP reaction which are used during primer screening (Figure 1). Optimized RT-LAMP condition for finally selected two primer sets, namely “Nsp3_1-61” and “Nsp3_2-24”, are representatively provided. We also adapted leuco crystal violet (LCV) colorimetric detection method optimized for Bst 3.0 buffer system (Miyamoto *et al.*, 2015). A commercially available colorimetric RT-LAMP premix uses pH sensitive dye so that the premix is not compatible with viral genome extraction methods which affect pH of weakly buffered reaction solution (Lalli *et al.*, 2020). However, LCV method is compatible with such viral genome extraction methods because the color change depends on dsDNA product.

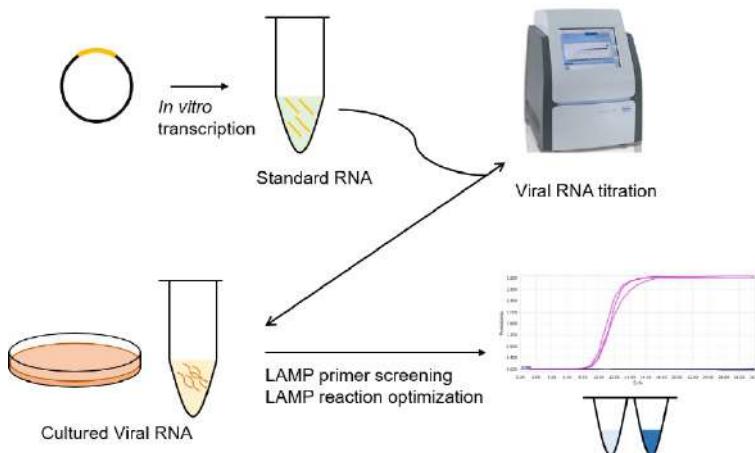


Figure 3. Protocol overview

Materials and Reagents

1. LightCycler® 8-Tube Strips, Clear (Roche, catalog number: 06327672001)
2. LightCycler® 480 Multiwell Plate 96, Clear (Roche, catalog number: 05102413001)
3. AccuPower PCR PreMix (-dye) kit (Bioneer, catalog number: K-2016)
4. Agarose (Bio Basic, catalog number: D0012)
5. 50x TAE (Biosesang, catalog number: T2002)
6. MEGAscript™ T7 Transcription Kit (Invitrogen, catalog number: AM1334)
7. Formaldehyde, 37% w/w aq. Soln. (Alfa Aesar, catalog number: A16163)
8. 10x MOPS (Bioneer, catalog number: C-9031)
9. SYBR™ Green II RNA Gel Stain (Invitrogen, catalog number: S7564)
10. RiboRuler Low Range RNA Ladder (Thermo Scientific, catalog number: SM1831)
11. QuantiFluor® RNA System (Promega, catalog number: E3310)
12. 1 M Tris-HCl, pH 7.5 (Biosesang, catalog number: T2016-7.5)
13. 0.5 M EDTA, pH 8 (Biosesang, catalog number: E2002)
14. QIAamp Viral RNA Mini Kit (Qiagen, catalog number: 52906)
15. Luna® Universal Probe One-Step Reaction Mix (NEB, catalog number: E3006)

16. DEPC treated water (Biosesang, catalog number: W2004)
17. WarmStart® Colorimetric LAMP 2x Master Mix (NEB, catalog number: E1800)
18. SYTO™ 9 (Invitrogen, catalog number: S34854)
19. Bst 3.0 DNA polymerase (NEB, catalog number: M0374, The product includes Isothermal Amplification Buffer II and Magnesium Sulfate solution.)
20. dNTP Mixture, 10 mM ea. (Enzyomics, catalog number: N002)
21. SuperScript™ IV Reverse Transcriptase (Invitrogen, catalog number: 18090050)
22. Crystal Violet (Sigma, catalog number: C0775)
23. Sodium Sulfite (Sigma, catalog number: S0505)
24. β-Cyclodextrin (Sigma, catalog number: C4767)
25. pET21a plasmid containing SARS-CoV-2 *Envelope* gene (Bionics, custom order)
26. T7 promoter primer (5'-AATACGACTCACTATAG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') (Macrogen) (Table 1)
27. Denaturing agarose gel (1%, 100 ml) (see Recipes)
28. 20 μM SYTO 9 (see Recipes)
29. 5x LCV solution (see Recipes)

Table 1. Primer sequences used for RT-qPCR and RT-LAMP

Primer set	Primer	Sequence (5'-3')	Note
E_Sarbeco	Forward	ACAGGTACGTTAACAGTTAACAGCGT	(Corman et al., 2020)
	Reverse	ATATTGCAGCAGTACGCACACA	
	Probe	FAM-ACACTAGCCATCCTACTGCGCTTCG-BHQ1	
Nsp3_1-61	F3	GGAATTGGTGCCACTTC	
	B3	CTATTCACTTCAATAGTCTGAACA	
	FIP	CTTGTGACCAACAGTTGTTGACTTCAACCTGAAGAAGAGCAA	
	BIP	CGGCAGTGAGGACAATCAGACACTGGTGTAAAGTTCCATCTC	
	LF	ATCATCATCTAACCAATCTCTTC	
	LB	TCAAACAATTGTTGAGGTTCAACC	
Nsp3_2-24	F3	TGCAACTATAAGGCCACG	
	B3	CGTCTTCTGTATGGTAGGATT	
	FIP	TCTGACTTCAGTACATCAAACGAATAAACCTGGTGTACGTTGTC	
	BIP	GACGCGCAGGGAATGGATAATTCCACTACTTCTTCAGAGACT	
	LF	TGTTTCAACTGGTTTGCTCCA	
	LB	TCTTGCCTGCGAAGATCTAAAAC	

Equipment

1. Mupid-One (ADVANCE, catalog number: AD160)
2. ChemiDoc™ Touch Imaging System (Bio-Rad, catalog number: 1708370)
3. Quantus™ Fluorometer (Promega, catalog number: E6150)

4. LightCycler® 96 Instrument (Roche, catalog number: 05815916001)

Software

2. Polynucleotide Molecular Weight Calculator (Developed by Andrew Staroscik, <http://scienceprimer.com/nucleotide-molecular-weight-calculator>)
3. LightCycler® 96 Software (Roche)

Procedure

A. Preparation of *in vitro* transcribed RNA standards for SARS-CoV-2 *E* gene

1. Prepare PCR product as template for *in vitro* transcription: Run PCR with AccuPower® PCR PreMix (-dye) kit as manufacturer's instructions. Use pET21a plasmid containing SARS-CoV-2 *Envelope* gene as template and T7 promoter primer and T7 terminator primer.
2. Perform *in vitro* transcription using MEGAscript™ T7 Transcription Kit as manufacturer's instructions.
3. Confirm RNA product by subjecting a portion to formaldehyde-MOPS denaturing agarose gel electrophoresis. The same volume of RNA loading buffer II included in MEGAscript™ T7 Transcription Kit was added and heated for 10 min at 65°C for sampling. Post-stain gel with 2× SYBR green II in 1× TAE and obtain image using ChemiDoc™ Touch Imaging System (Figure 2).

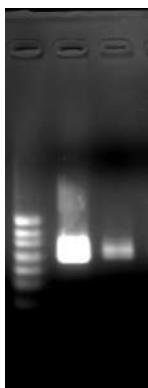


Figure 4. Agarose gel image of *in vitro* transcribed SARS-CoV-2 *E* gene RNA. Two 10-fold serial dilutions are loaded. Ladder sizes are corresponding to 1,000, 800, 600, 400, 300, and 200 bp from up.

4. Measure RNA concentration with Quantus™ Fluorometer.
5. Calculate RNA copy number.
 - a. Obtain molecular weight (M.W.) of the transcribed RNA (corresponding sequence: 5'-GGGGAAUUGUGAGCGGAUAACAUUCCCCUCUAGAAAUAUUUUGUUUAACUUUAAGAAGGAGAUUAACAUUAUGUACUCAUUCGUUUCGGAAGAGACAGGUACGUUAAUAGUUAUAGCGUACUUCUUUUUCUUGCUUUCGUGGUAUUCUUGCUAGGUACACUAGCCAUC

UUACUGCGCUUCGAUUGUGUGCGUACUGCUGCAAUAUUGUUACGUGAGUCUUGUA
AAACCUUCUUUUUACGUUUACUCUCGUGUAAAAACUGAAUUCUUCUAGAGUUCU
GAUCUUCUGGUCUAACUCGAGCACCCACCACUGAGAGAUCCGGCUGCUAAC
AAAGCCCCAAAGGAAGCUGAGUUGGCUGCCACCGCUGAGCAUAACUAGC-3')
using Polynucleotide Molecular Weight Calculator, or using following formula where "#N" represents the number of each nucleotide.

$$\text{M.W.} = (\#A \times 329.2) + (\#U \times 306.2) + (\#C \times 305.2) + (\#G \times 345.2) + 159$$

- b. Calculate copy number using following formula:

$$\text{RNA (copies}/\mu\text{l}) = \left(\frac{\text{concentration(ng}/\mu\text{l)}}{\text{molecular weight(g/mol)}} \times 10^{-9} \right) \times 6.022 \times 10^{23}$$

6. Make 10-fold serial dilutions in TE buffer as RT-qPCR standards.

B. Viral RNA Preparation and Titration Using *E* gene

1. Extract viral RNA from debris-free culture media with QIAamp Viral RNA Mini Kit following manufacturer's instructions.
Aliquot viral RNA extract and store at -80°C until use.
2. Titrate cultured viral RNA copy number by RT-qPCR
 - a. Prepare primer mix as follows: for 100 μl, mix 10 μl of forward and reverse primer, 5 μl of probe, 75 μl of DEPC treated water. Stock concentration of each primer/probe is 100 μM. Names and sequences of primers are listed in Table 1. Primer mix may be prepared in a batch and stored in -20 °C for later use.
 - b. Prepare master mix except template as follow: 7.5 μl of Luna® Universal Probe One-Step Reaction Mix, 0.75 μl of Luna® WarmStart® RT Enzyme Mix, 0.6 μl of primer mix, and 4.15 μl of DEPC treated water per reaction.
 - c. Aliquot 13 μl of master mix to each well of PCR tube or plate.
 - d. Add 2 μl of template (viral RNA dilutions, *in vitro* transcribed RNA standards or no template control) to each well. Close lid of PCR tube or cover film on plate.
 - e. Run PCR reaction as following temperature and time setting: 10 min at 55°C, 1 min at 95°C, and 45 cycles of 10 s at 95°C and 30 s at 60°C.
 - f. Copy number of viral RNA can be calculated by substituting threshold cycle value (Ct) to the equation of standard curve of following form:

$$\text{Ct} = \text{slope} \times \log_{10}(\text{template copy number}) + (\text{y-intercept})$$

Standard curve generation and sample copy number calculation can be done using each software coupled with real-time PCR instrument.

C. RT-LAMP reaction

1. Template viral RNA may be prepared from cultured virus or biological specimen from COVID-19 patients. Selection of proper RNA extraction method is up to performers.
2. Prepare 10× LAMP primer mix as follows: for 100 µl, mix 16 µl of FIP/BIP, 2 µl of F3/B3 primers, 4 µl of LF/LB primers and 56 µl of DEPC treated water. Stock concentration of each primers is 100 µM. Sequences of primers are listed in Table 1. The LAMP primer mix may be prepared in a batch and stored in -20°C for later use.
3. Prepare RT-LAMP reaction mix. Master mix except template can be prepared in a batch.
 - a. For LCV method, Reaction components, stock concentration and volume to use are as listed in Table 2.
 - b. When using WarmStart® Colorimetric LAMP 2× Master Mix, prepare reaction mix as follows: 2 µl of template, 1.5 µl of 10× LAMP primer mix, 0.3 µl of 20 µM SYTO9, 7.5 µl of LAMP mix, and 3.7 µl of DEPC treated water.

Table 2. Components and volume used (µl) for RT-LAMP with designated LAMP primer sets

Component	Nsp3_1-61	Nsp3_2-24
Template	2	2
10× LAMP primer mix	1.5	1.5
10× Isothermal Amplification Buffer II	1.5	1.5
MgSO ₄ (100 mM)	0.6	0.9
dNTPs (10 mM ea.)	1.5	2.1
5× LCV solution	3	3
SYTO 9 (20 µM)	0.3	0.3
<i>Bst</i> 3.0 (8 U/µl)	0.75	0.75
SuperScript IV (200 U/µl)	0.1	0.1
DEPC treated water	3.75	2.85

4. Aliquot 13 µl of reaction mix and 2 µl of template to each well of PCR tube or plate and close lid or cover film.
5. Run isothermal amplification. Following example settings are for the LightCycler® 96 instrument SYTO 9 as fluorescent dye.
 - a. Select SYBR Green I and set “Integration Time” to 1 s.
 - b. Set cycling condition to 59 s at 65°C for Nsp3_1-61 primer set or 69 °C for Nsp3_2-24 primer set. Use 65°C for reaction with WarmStart® Colorimetric LAMP 2x Master Mix. Set 59 s of incubation time and 1 s of data acquisition time for each cycle to make actual incubation time per cycle as 1 min. Set number of cycles to the desired incubation time in minutes (Figure 3).

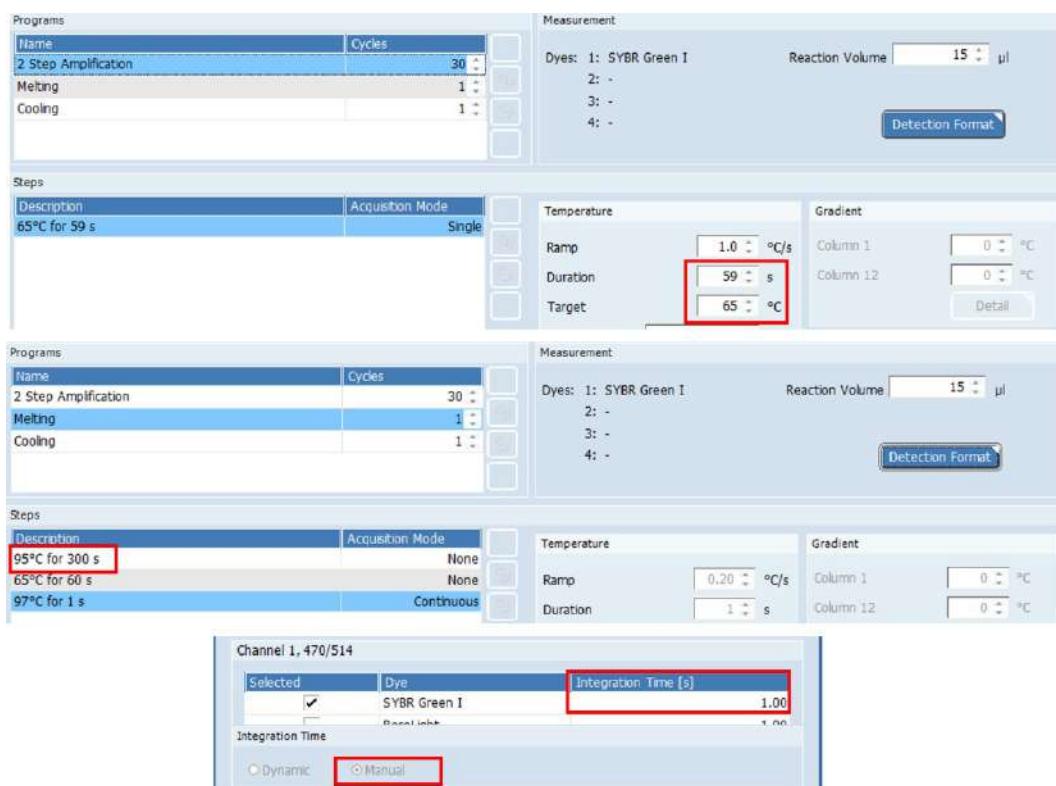


Figure 5. Example settings for the primer set Nsp3_1-61 using LightCycler® 96 software

Data analysis

1. Real-time amplification of LAMP reactions can be observed via LightCycler 96 software or other program accompanied with each qPCR machines. Sigmoidal amplification curve is shown for samples with positive result while no amplification is shown for negative samples or samples with negative results. Non-specific amplification curve may be observed with significantly delayed threshold time and/or shifted melting temperature (Figure 4).
2. For tests for which WarmStart Colorimetric LAMP 2× Master Mix is used, samples with positive result show yellow color while samples with negative result show pink color. For tests to which LCV method is adapted, samples with positive result show clear blue color from blueish transparent color (Figure 5).

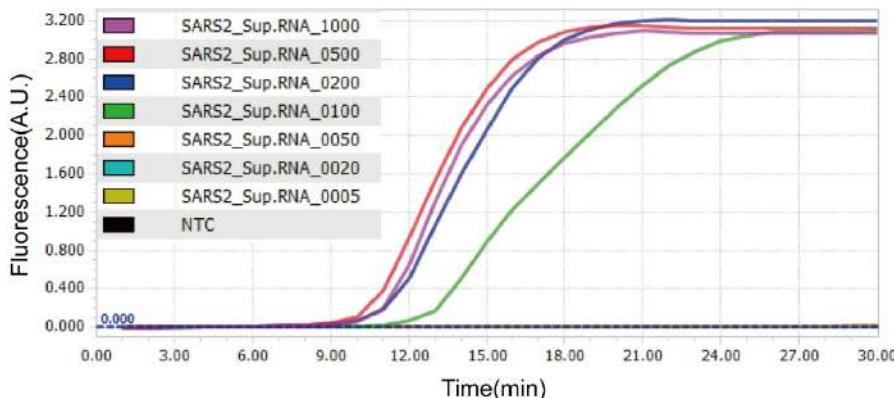


Figure 6. Amplification curves of RT-LAMP using Nsp3_1-61 primer set on LightCycler 96 system. Representative amplification curves of one replicate sample with designated RNA copies are shown. NTC, No Template Control.

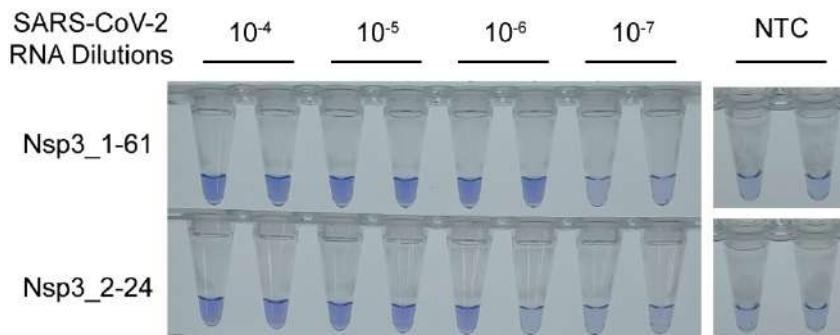


Figure 7. The color changes of LCV method for RT-LAMP. LCV color changes to blue by positive RT-LAMP amplification. RT-LAMP reaction was performed for 60 min at 69°C with the half amount of SuperScript IV reverse transcriptase described in the main protocol as the result is from an experiment during reaction optimization. NTC, No Template Control.

Notes

1. Here we targeted *E* gene for RT-qPCR titration while LAMP primer targets *Nps3*. Therefore titrated RNA copy number does not perfectly match with LAMP target copy number.
2. Denaturing agarose gel for RNA electrophoresis should be prepared in fume hood as vapor with formaldehyde is toxic. It would be better to do electrophoresis in fume hood too.
3. *In vitro* transcription usually give micrograms of RNA products.
4. Same as or more than seven points with 5 to 10 fold serial dilution are generally recommended for standards of RT-qPCR. We used 10^8 to 10^0 copies per reaction and no template control (NTC) for standard curve generation. Triplicate for each dilution is sufficient.
5. While we obtained about 10^7 copies/ μ l concentration from cultured virus, the number may differ by incubation period and virus species. Non-infected cells work as the negative control for viral infection yet the derived RNA is not necessarily included for RT-qPCR as no template control can

serve as the negative control of RT-qPCR.

6. When preparing a master mix for RT-qPCR and LAMP/RT-LAMP in bulk for a batch of experiment, add extra volume to prevent shortage of the master mix from pipette error or other reasons. For 15 μ l reaction described in this protocol, 0.5 to 1 extra amount for < 20 reactions or about 10% extra amount for more reactions is usually enough.
7. For RT-qPCR and real-time RT-LAMP, other qPCR instruments than LightCycler® 96 Software can be used. For real-time RT-LAMP, the time required for measuring fluorescence should be accounted for setting incubation time and cycle scheme and for the calculation between Ct and threshold time. For example, Bio-Rad CFX systems need rather unpredictable data acquisition time as the system's detection unit scan each well one by one.
8. A melting step may be added to the RT-LAMP reaction since non-specific amplifications may be distinguished from their melting curves. Addition of an incubation step for 5 min at 95 °C between amplification and melting steps is rather recommended for polymerase inactivation.
9. When using LCV method for colorimetric detection, color varies by temperature. In high temperature, color of reaction mixture may turn to blue without nucleic acid amplification. Color of the mixture will back to normal in room temperature (18-25°C) within 5 min.
10. The sensitivity of LAMP is bottlenecked by formation of 'dumbbell intermediate' and it is usually lower than that of PCR. However, LAMP is highly vulnerable to cross contamination because LAMP yield is high and the bottleneck is not applied. Therefore, we strongly discourage to open reaction tube after LAMP reaction. Otherwise, using separated space for analysis of reaction product (e.g., agarose gel electrophoresis) is recommended.
11. Typical LAMP optimization includes changing concentration of dNTPs or Mg²⁺. We suggest to test each concentration in combination because dNTPs can chelate Mg²⁺.
12. Since EDTA in TE buffer chelate Mg²⁺ ion, using DEPC treated water for RNA elution and dilution may be beneficial when testing larger volume of template.

Recipes

1. Denaturing agarose gel (1%, 100 ml)
1g agarose
10 ml 10× MOPS
18 ml 37% formaldehyde
72 ml DEPC treated water
2. 20 μ M SYTO 9
Dissolve 5 mM SYTO 9 original stock in DEPC treated water with 1/250 dilution. Aliquot in small volume (~100 μ l) and store at -20°C
3. 5× LCV solution
10.20 mg Crystal Violet
378.12 mg Sodium Sulfite
283.75 mg β -Cyclodextrin

Dissolved in 50 ml DEPC treated water

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Competing interests

The authors declare no conflict of interests.

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