Production, titration, neutralisation and storage of 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-titer viral pseudotype particles with the SARS-CoV-2 spike protein (and D614G variant) on a lentiviral vector core, and use for neutralization assays in target cells expressing ACE2 and TMPRSS2. It additionally provides detailed instruction on substituting in new spike variants via gene cloning, and storage/shipping considerations for wide deployment potential. Results obtained with this protocol show that SARS-CoV-2 pseudotypes can be produced at equivalent titers to SARS-CoV and MERS-CoV pseudotypes, that they can be neutralized by human convalescent plasma and monoclonal antibodies and that they can be stored at a range of laboratory temperatures and lyophilized for distribution.

Keywords: SARS-CoV-2 coronavirus, lentiviral pseudotype, virus neutralization

Introduction

The design, production and use of pseudotyped viral particles (PV) is widely established for many emerging viruses, and applications in the fields of serology, sero-surveillance, mAb potency/breadth, and vaccine immunogenicity testing are manifold (Carnell 2015). 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. They are produced by co-transfection of plasmids into 293T producer cells. 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 such as SARS-CoV-2 the causative agent of COVID-19. They are readily upgradable for the study of new virus variants (for example D614G) via in-house or commercial site directed mutagenesis or swapping out of the full Spike (S) cassette. Figure 1 shows a cartoon of the lentiviral SARS-CoV-2 PV production process directed by three plasmid co-transfection in human 293T cells. These SARS-CoV-2 PVs were first used for the detection of neutralizing antibodies to SARS-CoV-2 to determine population exposure in blood donors during the first UK lockdown period in the UK (Thompson 2020) and the study was made available immediately via the medRxiv pre-print repository with versioning (https://www.medrxiv.org/content/10.1101/2020.04.13.20060467v1?versioned=true).

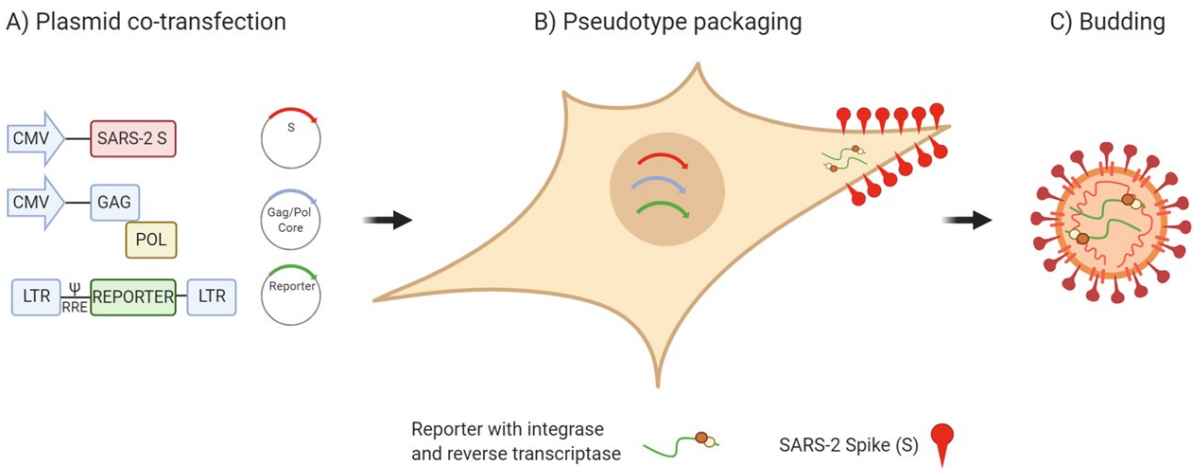


Figure 1. Cartoon representation of the production of SARS-CoV-2 pseudotypes. HEK293T/17 cells are transfected with three plasmids (Lentiviral vector incorporating luciferase reporter, packaging construct and SARS-CoV-2 spike expression plasmid) for the production of SARS-CoV-2 Spike bearing lentiviral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

Materials and methods

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 (10cm 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 3ml, (Fisher Scientific, Thermo Scientific™, catalog number: 14955457)
6. 0.45 um 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. HEK 293T/17 cells (ATCC, catalog number: CRL-11268)
10. Plasmids
    a. Spike plasmid: pCAGGS-SARS-CoV spike (CFAR, catalog number: 100976)
    b. Lentiviral vector expressing firefly luciferase: pCSFLW (or pCSGW for GFP PV)
    c. Second-generation lentiviral packaging construct plasmid: p8.91 (expresses gag, pol and rev)
    d. Host cell entry receptor angiotensin-converting enzyme 2 (ACE2) expression plasmid: pCDNA3.1+-ACE2 (Simmons 2004)
    e. Coronavirus Spike (S) protein priming transmembrane serine protease 2 (TMPRSS2) expression plasmid: pCAGGS-TMPRSS2 (Bertram 2010)

    Note: Information on the plasmids above can be found in Temperton 2005 and Carnell 2015.
11. 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)
12. Gibco Reduced Serum media Opti-MEM® (Thermo Fisher Scientific, catalog number: 51985034)
13. FuGENE® HD Transfection Reagent, 1ml (Promega, catalog number E2311)
14. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Pan-Biotech, catalog number: P04-36500)

15. Trypsin-EDTA (0.05%), phenol red (Pan-Biotech, catalog number: P10-040100)
16. Positive control antibody (Research Reagent for Anti-SARS-CoV-2 Ab) that can neutralize the SARS-CoV-2 PV (NIBSC code 20/130)
17. COVID-19 human convalescent plasma panel (NIBSC code 20/118)
18. Monoclonal antibodies that can neutralize the SARS-CoV-2 PV (Native Antigen MAB12443 and MAB12444)
19. 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, generic
3. Pipettes (Gilson, model: PIPETMAN® Classic, P2, P20, P200 and P1000 or equivalent)
4. Multichannel pipette (Glison, model PIPETMAN L Multichannel P12 20-200ul or equivalent)
5. Fisherbrand™ Sterile Polystyrene Disposable Serological Pipets 5ml and 10ml in 1/10ml, Sterile, Plugged, Individually Wrapped (Fisher Scientific, Thermo Scientific™, catalog number: 1367610H and 1367610J)
6. Portable Pipet-Aid® XP Pipette Controller (Drummond Scientific Company, catalog number: 4-000-101 or equivalent)
7. 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)
8. Plate centrifuge (ELMI, model: SkyLine CM-6MT)
9. GloMax® Navigator Microplate Luminometer (Promega, model: GloMax® Navigator)
10. PC or Mac with Microsoft Excel (Microsoft®) and GraphPad Prism® (GraphPad Software).

Procedures A-G

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 x 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.

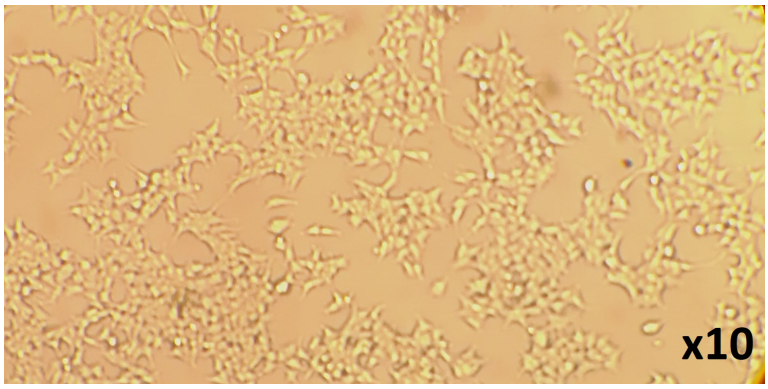


Figure 2. Example of the confluency expected prior to transfection of HEK293T/17 cells with plasmids

2. Day 2: DMEM/10% FBS/1% P/S and Opti-MEM® should be pre-warmed to 37C 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-2 or pCAGGS-SARS-CoV-2 (D614G) 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 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 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 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 37C, 5% CO_2 for 44-52 h. In our hands incubation times in this range result in equivalent final PV production RLU titers.

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 -80C 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. We have observed that cells in poor health after first harvest result in significantly lower PV production RLU titers 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 S PV control).

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

Note: SARS-CoV-2 virus host cell entry depends on receptor ACE2 and serine protease TMPRSS2 for S protein priming (Hoffmann 2020). HEK293T/17 are transfected with ACE2 and TMPRSS2 plasmids in order 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 neutralization assay is planned.

1. HEK 293T/17 cells should be seeded into a 10cm cell culture dish at a ratio that will deliver 70-90% confluence at the time of transfection. Typically seeding $2x10^6$ 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 37C 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 for transfection (DNA mix):
   pCDNA3.1+-ACE2 cell entry receptor: 2 ug
   pCAGGS-TMPRSS2 serine protease: 150 ng
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 37C, 5% CO_2 overnight. In our hands incubation times result in sufficient overexpression of cell entry receptors for SARS-CoV-2 PV entry.

C. SARS-CoV-2 PV Titration for the calculation of RLU/ml

Note: 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 24h prior to the titration 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 utilizes a ubiquitous receptor which results in high RLU titers in all cell lines tested.

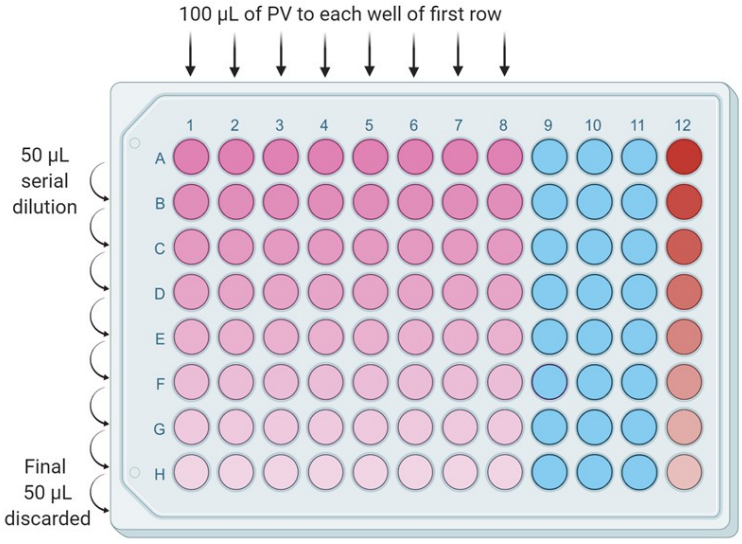


Figure 3. 96-well plate set-up for pseudotype titration. Serial dilution step showing addition of 100 µl of pseudotype virus supernatant to each well of row A and dilution of 50 µl taken from this well to row B. This process is continued to end of plate (row H) at which point the final 50 µl is discarded. 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).

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 row 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).
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 5 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 x 10^4 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 37C 5% CO_2.
10. Read plate using Bright Glo™ luciferase assay system on a GloMax® Navigator Microplate Luminometer (or equivalent).

Results

Data analysis

1. RLU readings from the luminometer are multiplied to get RLU/ml by the dilution factor of each well (20x, 40x, 80x, 160x, 320x, 640x, 1,280x, 2,560x). 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).

	ACE2 + PV	ACE2 only	Cell only	MPRSS2 + P	MPRSS2 on	Cell only	Cell only	+TMPRSS2	+TMPRSS2	Cell only	EK293T + P	EK293T only
20	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
40	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
80	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
160	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
320	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
640	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
1280	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
2560	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 + PV	ACE2 only	Cell only	MPRSS2 + P	MPRSS2 on	Cell only	Cell only	+TMPRSS2	+TMPRSS2	Cell only	EK293T + P	EK293T 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

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 8 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 titter in this experiemnt (3.1e8 RLU/ml) is achieved when both ACE2 and TMPRSS2 are present in the target cells (column 8).

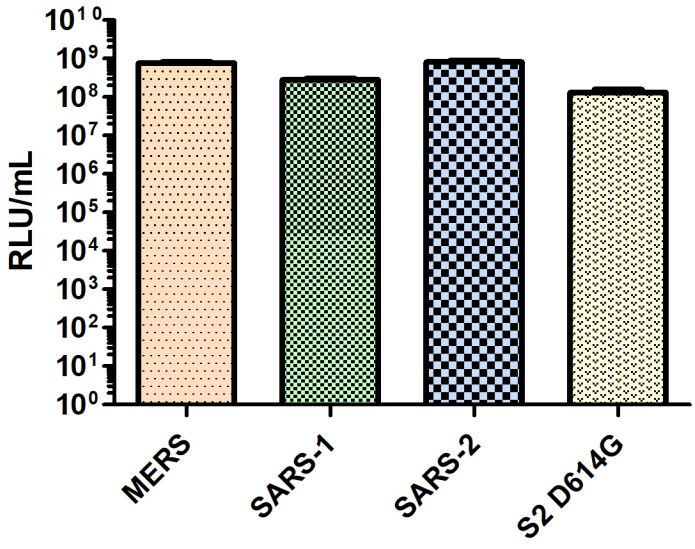


Figure 4. Production of SARS-CoV-2 PV (and D614G variant) and comparison with SARS-CoV (Temperton 2005) and MERS-CoV (Grehan 2015). RLU/ml production titters shown for MERS-CoV, SARS-CoV, SARS-CoV-2 and SARS-CoV-2 (D614G). SARS-CoV, SARS-CoV-2 and SARS-CoV-2 PV titrated on 293T/ACE2/TMPRSS2 cells, MERS-CoV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

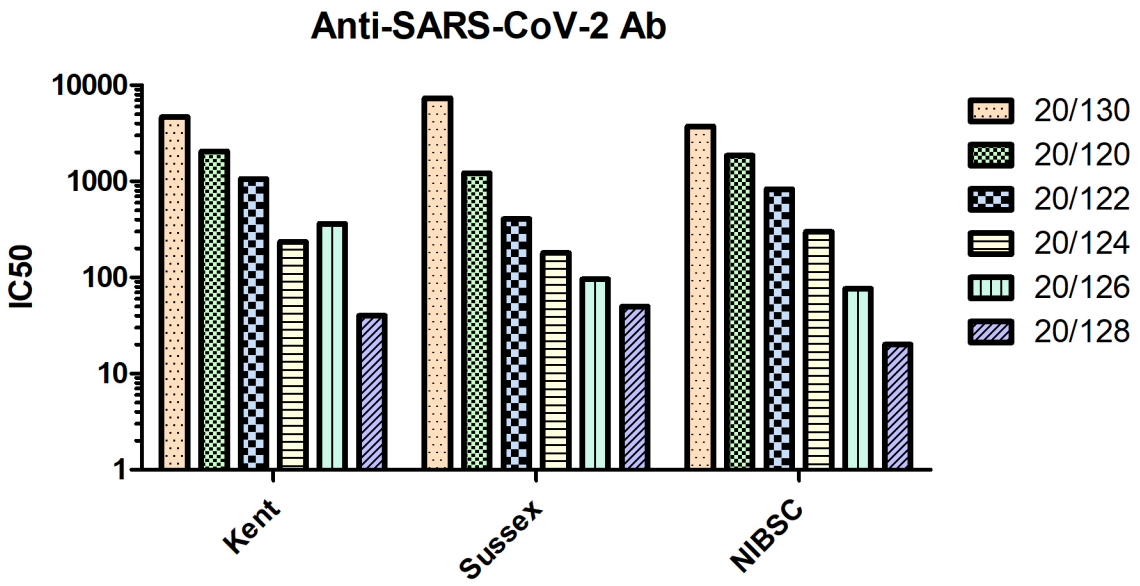
D. Pseudotype based neutralisation assay (pMN)

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 (example 5 µl convalescent sera or mAb at 10ug/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 x 10^6 RLU in 50 µl, with a total volume of 5 ml. For example, with an RLU/ml of 1 x 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 µl 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 37C 5% CO_2, 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:
    i. Remove culture media from plate.
    ii. Wash the plate twice with 2 ml of PBS to one side of the dish to avoid cell detachment and discard.
    iii. Add 2 ml of trypsin to the plate and put the plate into the incubator until the cells are detached.
    iv. 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.
    v. Count cells using TC20™ Automated Cell Counter or counting-chamber slide and add 1 x 10^4 cells in a total volume of 50 µl to each well.
12. Incubate the plate for 48-72 h at 37C 5% CO_2.
13. Read plate using Bright Glo™ luciferase assay system on a GloMax® Navigator Microplate Luminometer (or equivalent).
14. From the raw data provided by the luminometer, calculate the IC50 neutralizing antibody titers using the previously optimized protocol from our group which is currently being used by the NIBSC and other stakeholders (Ferrara 2018).



	VPU Kent	VPU Sussex	NIBSC
20/130	4646	7319	3721
20/120	2052	1220	1853
20/122	1063	407	827
20/124	235	180	299
20/126	362	96	76

20/128	<40	<50	<20

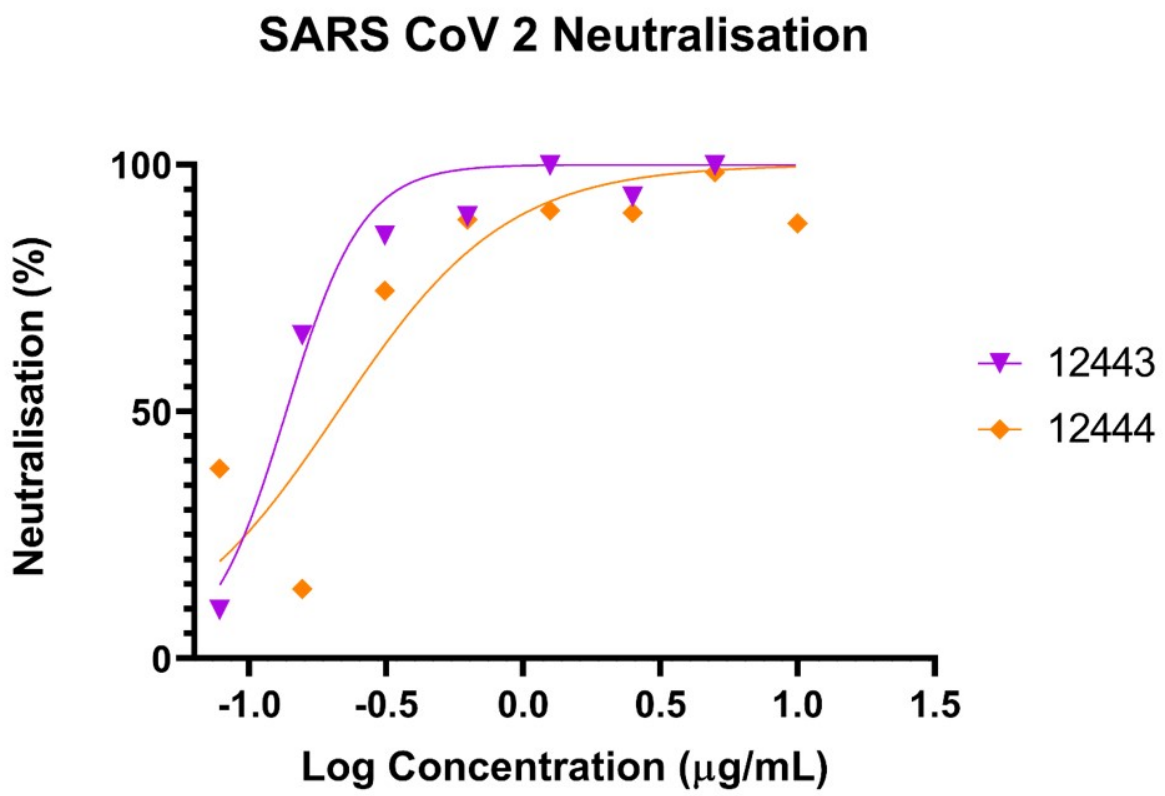


Figure 5. Neutralization of SARS-CoV-2 viral pseudotype entry into target cells (HEK 293T/17 expressing ACE2 and TMPRSS2) using reference plasma panel from NIBSC or neutralizing mAbs from Native Antigen. Top panel: bar chart showing IC50s for panel of convalescent plasma run in three different laboratories, Middle panel: IC50 data for panel of plasma run in three different laboratories, Bottom panel: mAb neutralization 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 <24hr, UK to EU shipment will be <72hrs. For international destination shipments that may >72hrs, dry ice shipment or lyophilization is recommended.

1. Place 8 aliquots of 100 ul of SARS-CoV-2 PV (prepared above) at different storage temperature for 24h or 72h prior to titration (2 aliquots each were kept at RT, +4C, -20C or -80C).

2. After 24hr, add 4 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 72hr, add the remaining 4 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.

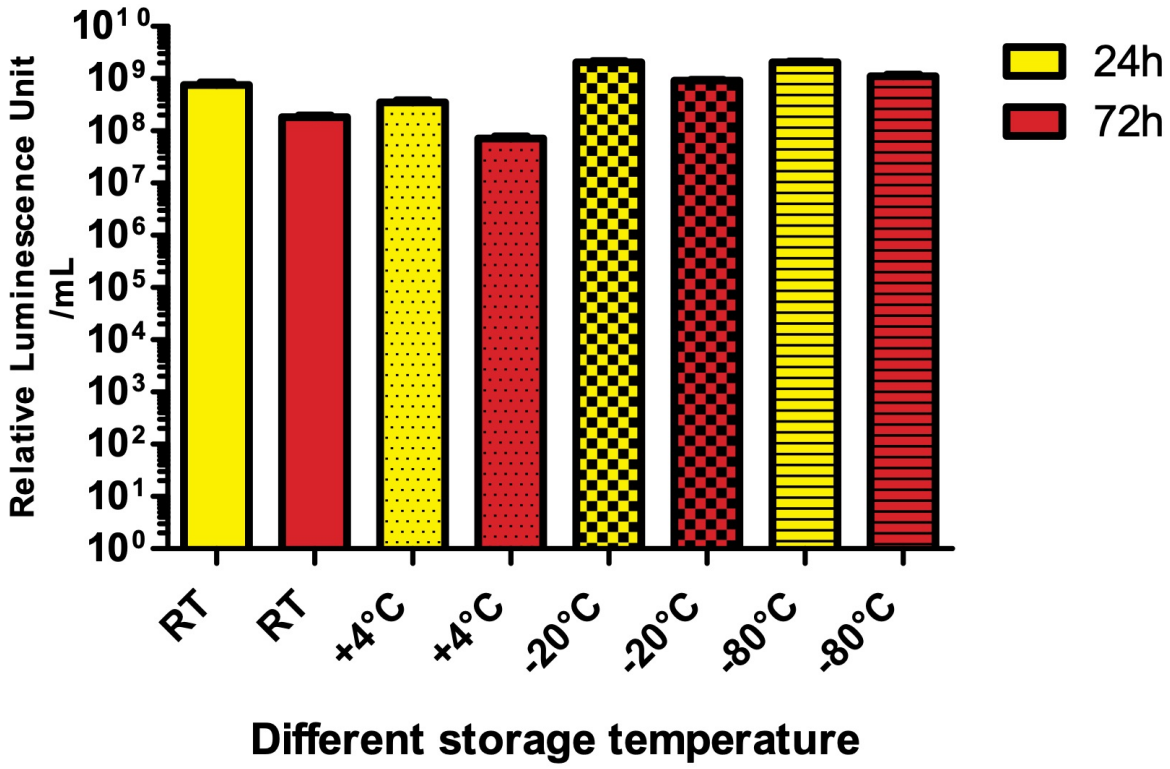


Figure 6. Short term storage of SARS-CoV-2 PV at different laboratory temperatures. PV were stored at RT, +4C, -20C and -80C for 24 or 72hr and then titrated. The PV aliquots kept at -80°C act as the positive control for titration experiment. -80C is routinely used for long term storage of PV.

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 2014). This methodology is applicable equally to coronavirus pseudotypes.

Materials and Reagents

1. Low surface tension polypropylene 1.5ml microtubes (Simport, catalog number: T330-7LST)
2. Sucrose (Sigma-Aldrich, catalog number: S0389)
3. Dulbecco's Phosphate Buffered Saline (DPBS; Pan Biotech, catalog number: P04-361000)

Equipment

1. FreeZone 2.5L freeze dryer (LabConCo, catalog number: 7670520)
2. Sample drying chamber (LabConCo, catalog number: 7318700)
3. Rotary Vane Vacuum pump 117 (LabConCo, catalog number: 7739402)
4. Plastic microtube rack (Thermo Fisher Scientific, catalog number: 8850)
5. Thermo-humidity meter (Thermo Fisher Scientific, catalog number: 11536973)

Procedure

1. Dissolve sucrose in DPBS to make a 1M cryoprotectant solution. Syringe sterilize 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 5s.
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 1hr.
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, wait until series of orange lights have lit up until final green light is illuminated (typically 0.035 mBar/3.5Pa). Leave overnight (16-20hrs). Note: samples should not bubble or move up microtubes during lyophilization (see Figure 7).
10. Open top valve slowly to equalize 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.

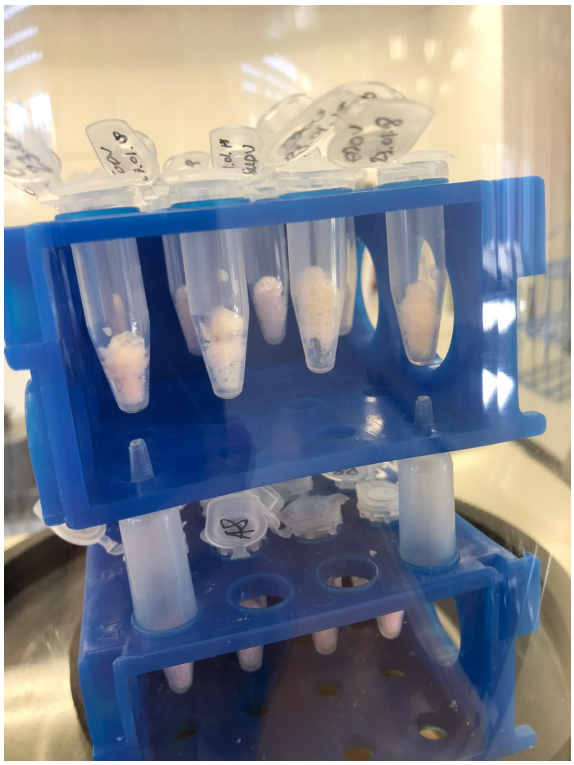


Figure 7. Lyophilized SARS-CoV-2 PV pellets within the sample drying chamber.

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

Materials and Reagents

1. Spike plasmid: pCAGGS-SARS-CoV spike (CFAR, catalog number: 100976)
2. Eukaryotic expression vector recipient plasmids: pcDNA3.1, pI.18, pCAGGS
3. Microtube (1.5ml, 0.5ml), generic
4. Thin-walled PCR microtubes (0.2ml), generic
5. Anza enzyme kit (Thermo Fisher Scientific, catalog number: IVGN3006)
6. Nuclease-free water (Thermo Fisher Scientific, catalog number: R0582)
7. QIAquick PCR Purification kit (Qiagen, catalog number: 28104)
8. QIAquick Gel Extraction kit (Qiagen, catalog number: 28704)
9. Tris Acetate EDTA (TAE) buffer (50x concentrate; (Thermo Fisher Scientific, catalog number: B49)
10. Ultrapure Agarose (Thermo Fisher Scientific, catalog number: 16500100)
11. SYBR Safe DNA gel stain (Invitrogen, catalog number: S33102)
12. GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific, catalog number: SM0313)
13. Luria Broth (LB) and LB agar powder (Sigma-Aldrich, catalog numbers: L3022 & L2897)
14. Subcloning efficiency *E. coli DH5α* cells (Invitrogen, catalog number 18265017)
15. DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, catalog number: K1081)

16. Monarch plasmid miniprep kit (New England Biolabs, catalog number T1010S) or alternative.

Equipment

1. Water bath or heat block, generic
2. Microwave, generic
3. Powerpack, generic
4. Gel electrophoresis tank, generic
5. Micro centrifuge (model, Sorvall Legend: Thermo Fisher Scientific)
6. UV transilluminator (Uvitech/Sigma, catalog number: Z363677)
7. Gel imaging system, generic
8. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, catalog number: ND-2000)
9. Dry 37C incubator, generic
10. Shaking 37C incubator, generic
11. Thermocycler (model, Prime: Techne, catalog number: 5PRIMEG/02)

Procedure

1. Separately restriction digest donor (2µg) and recipient plasmids (1µg) using appropriate enzymes according to manufacturer's instructions (cut either side of S gene, but not internally). For the former use Red Buffer (with gel loading dye), for the latter use Standard Buffer and nuclease free water for both.
2. Pour a 50ml 1% TAE Ultrapure agarose gel, adding 50µL SYBR Safe when microwaved gel mix cools to ~50C.
3. Load donor plasmid digest (and DNA ladder) onto gel and run for 1hr at 80V
4. Place gel on transilluminator (70% intensity setting) to visualize DNA and carefully excise Spike gene gel fragment using a clean scalpel.
5. Purify gene with the QIAquick Gel Extraction kit according to manufacturer's instructions. Elute in nuclease free water.
6. Purify recipient plasmid with the QIAquick PCR Purification kit according to manufacturer's instructions. Elute in nuclease free water.
7. Measure concentration of purified recipient plasmid and Spike gene fragment via Nanodrop
8. Perform Anza ligation reaction according to manufacturer's instructions using 50ng of recipient plasmid and a 1:3 molar ratio of S gene DNA.
9. Transform ligation into Subcloning Efficiency *E. coli* cells according to manufacturer's instructions, using LB broth as a culture medium.
10. Prepare LB gar plates containing appropriate antibiotic (i.e., ampicillin, Sigma-Aldrich 1000x stock 100mg/ml solution catalog number A5354).
11. Plate out and spread each 100µl transformed cell culture on ampicillin agar plates. Incubate at 37 C overnight.
12. Pick discrete colonies onto a grid on a new ampicillin plate. Incubate 37 C overnight.

13. Pick cells from each colony into a separate 0.2ml microtube in 50 µl nuclease free water. Lyse cells in thermocycler at 94 C for 3 min.
14. 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, 50C for 1 min, 72C for 2 min], 72C for 5 min.
15. Run on agarose gel as above. Photograph gel with gel imaging system and identify positive clones with S gene insert (~3.75kbp)
16. Inoculate 5ml LB-Amp broth cultures with positive clone cells from grid. Incubate in 37 C shaking incubator overnight.
17. Purify S gene plasmid clone DNA using miniprep kit.
18. Sequence verify using commercial service (Eurofins for example)
19. Once verified the new SARS-CoV-S plasmid can be incorporated into the PV production protocol above (Procedure A).

Conclusions

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).

Efficient knock-down (neutralization) 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 5. 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.

Author contributions

Conceptualization, NT, GC, AS and CT; Methodology, CDG, AS, SS, DC, MM, MD, BA, BF, DH, MS, AS, ET, GC and NT; Software, CDG and MM; Validation, GC, EB, GM and CT; Formal Analysis, NT and CDG; Investigation, CDG, GC and NT; Resources EB, GM, ET, GC; Data Curation, NT and CDG; Writing – Original Draft Preparation, NT; Writing – Review & Editing, NT, CDG, SS, GM, EB, EW; Visualization, GC, CDG; Supervision, NT, SS, GC and CT; Project Administration, NT; Funding Acquisition, NT

Conflicts of Interest

The authors declare no conflicts of interest.

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References

Bertram S, Glowacka I, Blazejewska P, Soilleux E, Allen P, Danisch S, Steffen I, Choi SY, Park Y, Schneider H, Schughart K, Pöhlmann S. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J Virol. 2010 Oct;84(19):10016-25. doi: 10.1128/JVI.00239-10. Epub 2010 Jul 14. PMID: 20631123; PMCID: PMC2937781.

Carnell GW, Ferrara F, Grehan K, Thompson CP, Temperton NJ. Pseudotype-based neutralization assays for influenza: a systematic analysis. Front Immunol. 2015 Apr 29;6:161. doi: 10.3389/fimmu.2015.00161. PMID: 25972865; PMCID: PMC4413832.

Ferrara F, Temperton N. Pseudotype Neutralization Assays: From Laboratory Bench to Data Analysis. Methods Protoc. 2018 Jan 22;1(1):8. doi: 10.3390/mps1010008. PMID: 31164554; PMCID: PMC6526431.

Grehan K, Ferrara F, Temperton N. An optimised method for the production of MERS-CoV spike expressing viral pseudotypes. MethodsX. 2015 Oct 13;2:379-84. doi: 10.1016/j.mex.2015.09.003. PMID: 26587388; PMCID: PMC4625112.

Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020 Apr 16;181(2):271-280.e8. doi: 10.1016/j.cell.2020.02.052. Epub 2020 Mar 5. PMID: 32142651; PMCID: PMC7102627.

Mather ST, Wright E, Scott SD, Temperton NJ. Lyophilisation of influenza, rabies and Marburg lentiviral pseudotype viruses for the development and distribution of a neutralisation -assay-based diagnostic kit. J Virol Methods. 2014 Dec 15;210:51-8. doi: 10.1016/j.jviromet.2014.09.021. Epub 2014 Oct 5. PMID: 25286181.

Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc Natl Acad Sci U S A. 2004 Mar 23;101(12):4240-5. doi: 10.1073/pnas.0306446101. Epub 2004 Mar 9. PMID: 15010527; PMCID: PMC384725.

Temperton NJ, Chan PK, Simmons G, Zambon MC, Tedder RS, Takeuchi Y, Weiss RA. Longitudinally profiling neutralizing antibody response to SARS coronavirus with pseudotypes. Emerg Infect Dis. 2005 Mar;11(3):411-6. doi: 10.3201/eid1103.040906. PMID: 15757556; PMCID: PMC3298259.

Thompson C., N. Grayson, R.S. Paton, J Lourenco, BS Penman, L. Lee, V. Odon, J. Mongkolsapaya, S. Chinnakannan, W. Dejnirattisai, M. Edmans, A. Fyfe, C. Imlach, K. Kooblall, N. Lim, C. Liu, C. Lopez-Camacho, C. McInally, N. Ramamurthy, J. Ratcliff, P. Supasa, B. Wang, A. Mentzer, M. Turner, M.G. Semple, JK Baillie, ISARIC4C Investigators, H. Harvala, G.R. Screaton, N. Temperton, P. Klenerman, L.M. Jarvis, S. Gupta, P. Simmonds. Neutralising antibodies to SARS coronavirus 2 in Scottish blood donors – a pilot study of the value of serology to determine population exposure. medRxiv 2020.04.13.20060467; doi: https://doi.org/10.1101/2020.04.13.20060467

Thompson CP, Grayson NE, Paton RS, Bolton JS, Lourenço J, Penman BS, Lee LN, Odon V, Mongkolsapaya J, Chinnakannan S, Dejnirattisai W, Edmans M, Fyfe A, Imlach C, Kooblall K, Lim N, Liu C, López-Camacho C, McInally C, McNaughton AL, Ramamurthy N, Ratcliff J, Supasa P, Sampson O, Wang B, Mentzer AJ, Turner M, Semple MG, Baillie K; ISARIC4C Investigators, Harvala H, Screaton GR, Temperton N, Klenerman P, Jarvis LM, Gupta S, Simmonds P. Detection of neutralising antibodies to SARS-CoV-2 to determine population exposure in Scottish blood donors between March and May 2020. Euro Surveill. 2020 Oct;25(42):2000685. doi: 10.2807/1560-7917.ES.2020.25.42.2000685. PMID: 33094713; PMCID: PMC7651873