Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay

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Pseudotyped viruses are useful virological tools because of their safety and versatility. On the basis of a vesicular stomatitis virus (VSV) pseudotyped virus production system, we developed a pseudotyped virus-based neutralization assay against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in biosafety level 2 facilities. Compared with the binding antibody test, the neutralization assay could discriminate the protective agents from the antibody family. This protocol includes production and titration of the SARS-CoV-2 S pseudotyped virus and the neutralization assay based on it. Various types of samples targeting virus attachment and entry could be evaluated for their potency, including serum samples derived from animals and humans, monoclonal antibodies and fusion inhibitors (peptides or small molecules). If the pseudotyped virus stock has been prepared in advance, it will take 2 days to get the potency data for the candidate samples. Experience in handling cells is needed before implementing this protocol.
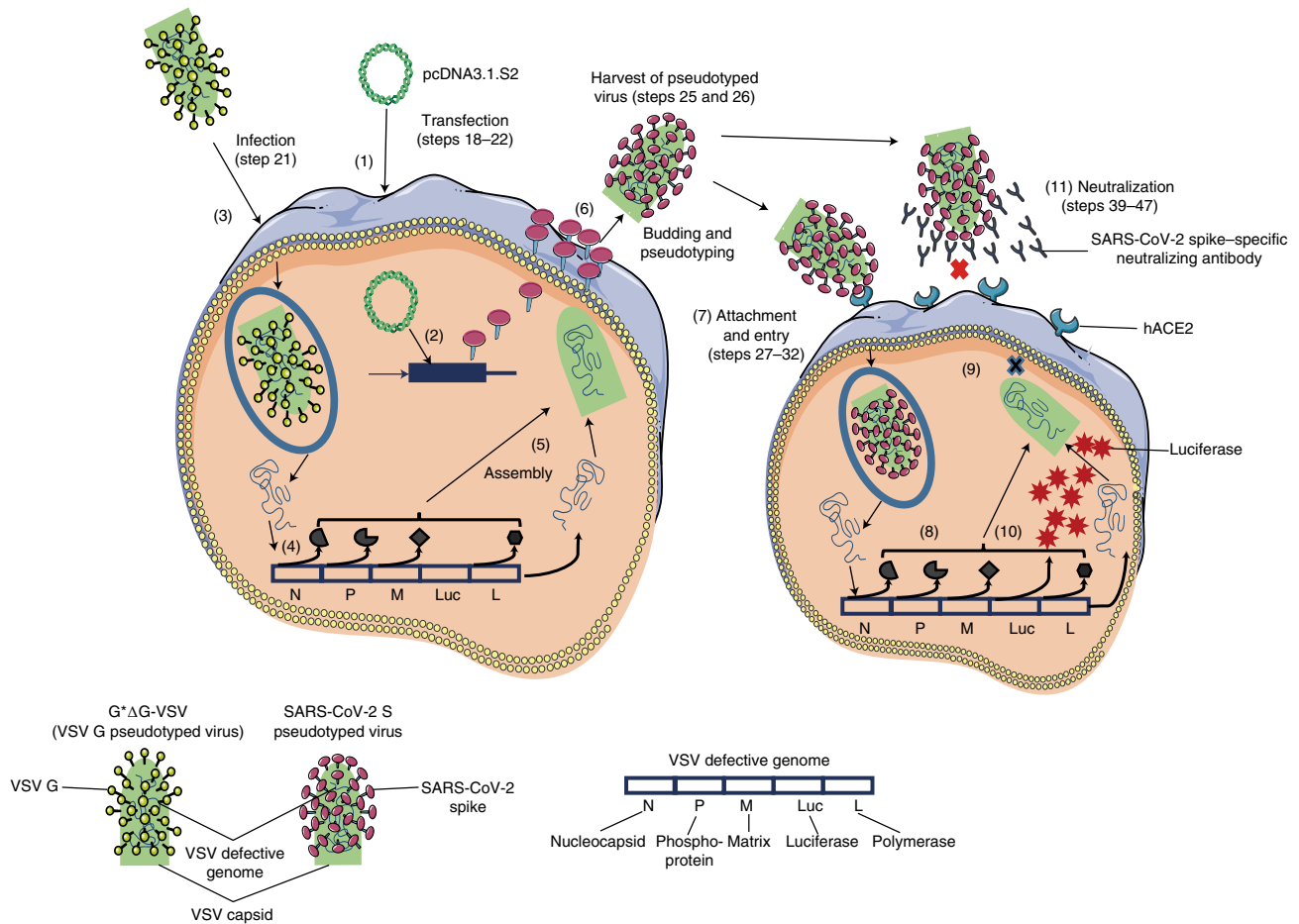
Introduction

More than 18 million cases of the coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), had been diagnosed globally by 6 August 2020. Therapeutics and vaccines for COVID-19 are urgently needed. Because of its high pathogenicity and infectivity, all test procedures employing live viruses to evaluate the products' potency have had to be handled under biosafety level (BSL) 3 facilities, which has hindered the development of products. Standardized in vitro potency methods are urgently needed to evaluate antiviral products in pre-clinical and clinical phases. In addition, detection of neutralizing antibodies against SARS-CoV-2 would be helpful to understand the status of the protective immune response among COVID-19 patients and asymptomatic cases[1].

SARS-CoV-2 is a single-stranded positive-sense RNA virus, belonging to the β-coronaviruses of the coronaviridae family[2]. It is one of the largest known RNA viruses with a genome length of ~29.8 kbp[3–5]. The first two-thirds of the genome, which consists of non-structural genes, mainly encodes enzymes related to virus replication, and the latter one-third sequentially encodes four structural proteins: spike protein (S), small envelope protein (E), matrix protein (M) and nucleocapsid protein (N)[6]. Among them, S protein contains the binding region of the virus receptor, which mediates the adsorption and entry of the virus into cells. The S protein of coronaviruses is the main protein that stimulates the immune system to produce neutralizing antibodies after the virus infects the body[7–10]. Various monoclonal neutralizing antibodies against the S protein of coronaviruses have been isolated and proven to be effective in animal models[11–14]. In addition, peptide fusion inhibitors[15] or small molecules that block host cell entry[16] have also been found to be effective in other coronavirus studies, which also targeted the S protein[7].Thus, the S protein is the main target for the design of SARS-CoV-2 vaccines and some therapeutic products.

Development of the protocol and production of a pseudotyped virus

The SARS-CoV-2 S pseudotyped virus is prepared by using a VSV pseudotyped virus packaging system[17], which has been previously used to generate various pseudotyped viruses, such as

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Fig. 1 | Schematic diagram of SARS-CoV-2 pseudotyped virus production and neutralization assay based on a VSV pseudotyped virus production system. 293T cells are transfected with SARS-CoV-2 S protein expression plasmid (pcDNA3.1.S2) (1), which is transcribed and expresses full-length S protein that is transferred to and anchored in the cell membrane (2). At the same time, 293T cells are infected with VSV G pseudotyped virus (G*ΔG-VSV), which enters the cells by using the VSV G protein on the surface of the particle and provides a defective VSV genome (3). After infection, the uncoated ΔG-VSV genome expresses all the enzymes and structural proteins (L, M, P and N) from the VSV genome except G protein (which has been replaced by a luciferase reporter gene, Luc) and completes partial genome replication (4). The expressed structural proteins and partially replicated genomes assemble into virus particles without an envelope (5), and the virus is released from the cells by budding, during which process the SARS-CoV-2 S protein expressed on the cell surface becomes the envelope protein of the pseudovirion. Thus, the pseudotyped virus with SARS-CoV-2 S protein incorporated on the envelope packages the defective VSV genome containing the reporter gene in its capsid (6). When these pseudotyped viruses are incubated with cells containing SARS-CoV-2 receptors, the pseudotyped viruses attach and enter the cells through the receptors on the cell surface (7). The pseudotyped viruses entering the cells will express all VSV proteins other than G protein, and these structural proteins will also be assembled into capsid protein particles with the replicated VSV defective genome (8). However, because there is no expression of surface proteins in this process, virus particles containing surface proteins cannot be formed (9). That is, the pseudotyped virus cannot replicate sequentially and has the ability of only a single-round infection. The VSV genome expresses the reporter gene Fluc, which can be detected after the addition of the substrate (10). If the pseudotyped virus is co-incubated with the sample containing neutralizing antibody or molecular inhibitors before cell infection, they will block the cell entry process (11). The number of blocked viruses can be determined by the results of chemiluminescence reduction, which reflects the level of neutralizing antibody or molecular inhibitors in the sample. (1)–(6) shows the process of production of SARS-CoV-2 pseudotyped virus. If the pcDNA3.1.S2 is replaced by pcDNA3.1.VSVG, the G*ΔG-VSV can be amplified following these procedures.

Ebola virus[18], Nipah virus[19], Hantaan virus[20], *Lyssavirus*[21] and Rift valley fever virus[17,22]. The backbone of the pseudotyped virus comes from the VSV virus, in which the *G* gene is replaced with the firefly luciferase (Fluc) reporter gene, and the S protein from SARS-CoV-2 is incorporated as the membrane protein on the surface of the VSV pseudotyped virus. The schematic procedure for preparation of SARS-CoV-2 pseudotyped virus and its subsequent use in a neutralization assay is shown in Fig. 1.

During the preparation of pseudotyped virus, 293T cells are transfected with the SARS-CoV-2 S protein expression plasmid (Fig. 1). After transfection, the plasmid is transcribed, and full-length S protein is expressed and becomes anchored in the cell membrane. At the same time, the cells are

infected with VSV G pseudotyped virus (G*ΔG-VSV), which enters the cells by using the VSV G protein on the surface of the particle and provides a defective VSV genome. After infection, the uncoated ΔG-VSV genome expresses the enzymes and structural proteins from the VSV genome, except G protein, and completes genome replication. The expressed defective genome assembles into virus particles without an envelope, and the virus is released from the cells by budding (Fig. 1). During this process, the SARS-CoV-2 S protein expressed on the cell surface is anchored in the membrane of VSV particles and becomes the envelope protein of the pseudovirion. Thus, the SARS-CoV-2 pseudotyped virus packaged the defective VSV genome, within which the G protein–expressing gene was replaced with a gene encoding Fluc. Bearing the S protein in the membrane, the pseudovirion has the characteristics of SARS-CoV-2 infection[23].

When the pseudotyped viruses are incubated with cells containing SARS-CoV-2 receptors, the pseudotyped viruses attach and enter the cells through the receptors on the cell surface. Both Huh-7 and Vero cells, which were used to isolate live virus[24], can be infected efficiently by SARS-CoV-2 pseudotyped virus[23]. The pseudotyped viruses entering the cells will express all VSV proteins other than G, and these structural proteins will be assembled to form the capsid that packages the defective VSV genome. However, because there is no expression of surface proteins in this process, virus particles containing surface proteins cannot be formed. Thus, the pseudotyped virus cannot replicate sequentially and has the ability of only a single-round infection. The VSV genome not only expresses its own proteins, but also expresses the reporter gene Fluc, which can be detected after the addition of the substrate. The amount of pseudotyped virus in infected cells can therefore be determined by detecting a luminescence signal on a luminometer. There is a linear correlation between the value of chemiluminescence signal and the amount of infected pseudotyped virus[23]. If the pseudotyped virus is co-incubated with a sample containing neutralizing antibody or molecular inhibitors before cell infection, they will block the cell entry process. The number of blocked viruses can be determined by the results of chemiluminescence reduction, which reflects the level of neutralizing antibody or molecular inhibitors in the sample (Fig. 1).

Overview of the procedure
Timeline
The SARS-CoV-2 pseudotyped virus neutralization test takes ~10 d from the beginning of virus preparation to the completion of the neutralization test. The preparation of plasmids used in the production of pseudotyped viruses takes 3 d (Steps 1–14). When the cells are ready, the production process of the pseudotyped virus takes 3 d (Steps 15–26). The preparation process of VSVG pseudotyped virus is basically the same as that of SARS-CoV-2 pseudotyped virus. The titration of pseudotyped virus takes 2 d (Steps 27–38). The pseudotyped virus neutralization test takes 2 d (Steps 39–48).

Plasmid preparation (Steps 1–14)
Two plasmids, pcDNA3.1.VSVG and pcDNA3.1.S2, are needed in the preparation of SARS-CoV-2 pseudotyped viruses. The former is used to amplify the VSV virus vector G*ΔG-VSV. G*ΔG-VSV can initially be obtained from Kerafast (EH1020-PM). However, it is only an aliquot with 300 μl of working stock, which is not enough to generate the SARS-CoV-2 pseudotyped virus. Thus, it is necessary to generate additional working stocks by amplifying G*ΔG-VSV in cells transfected with pcDNA3.1.VSVG before production of SARS-CoV-2 pseudotyped virus. To amplify the G*ΔG-VSV, the cell is infected with G*ΔG-VSV stock received from Kerafast that lacks the G protein gene, and at the same time the cells are transfected with the plasmid pcDNA3.1.VSVG to provide the G protein, which enables the G*ΔG-VSV to complete its replication cycle and generates more G*ΔG-VSV (Steps 17–26). The latter plasmid is prepared by inserting the SARS-CoV-2 *S* gene into the pcDNA3.1 plasmid by using BamH I and Xba I restriction sites, and used to deliver the SARS-CoV-2 S protein to the cell surface. To improve the expression efficiency of the SARS-CoV-2 S protein in mammalian cells, the *S* gene (GenBank: MN908947) was codon-optimized to enhance expression in mammal cells[23]. Both plasmids are used to transfect cells. To avoid the effect of endotoxin on transfected cells, we suggest that the plasmid be extracted with endotoxin-free reagents. The concentration of the extracted plasmids should be >0.5 mg/ml. The quality of plasmids evaluated by spectrophotometer should meet the general requirements of DNA plasmids (Step 13).
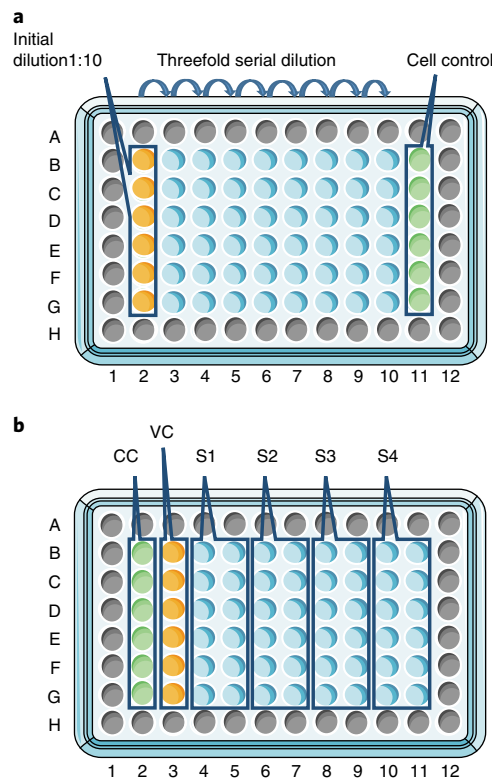
Fig. 2 | Plate design for pseudotyped virus titration (a) and neutralization assay (b). The gray wells indicate wells in which no samples were added, which are sealed with 250 μl of sterilized water per well to avoid the effect caused by evaporation of the edge-well culture medium. The green wells indicate the cell control (CC) wells, in which only cells with culture medium are added. In the plate in **a**, the orange wells are used as the initial dilution wells, in which pseudotyped viruses are diluted 10-fold as the first dilution. The blue wells are used as the serial dilution wells. In the plate in **b**, the orange wells are used as virus control (VC) wells, in which only pseudotyped viruses and cells are added with no samples. Blue wells are assigned as the sample wells, in which duplicates of each sample (S1–S4) are mixed with pseudotyped virus and incubated, followed by the addition of the cells.

Cell transfection and pseudotyped virus production (Steps 15–26)

SARS-CoV-2 pseudotyped viruses are prepared by SARS-CoV-2 S plasmid transfection providing membrane proteins on the surface of cells, and G*ΔG-VSV infection providing genomes of VSV (Fig. 1). In the same way, G*ΔG-VSV is amplified initially, by using VSVG plasmid instead of SARS-CoV-2 S plasmid. The first batch of cell supernatants are collected 24 h after infection and transfection, which is the first pseudotyped virus collection and can be stored at 2–8 °C for 24 h. After adding the same volume of complete culture medium to the cells for another 24 h, the cell supernatant is again collected, which is the second pseudotyped virus collection. Both the harvests of pseudotyped viruses are mixed, then divided into aliquots and cryopreserved at −80 °C, which is the bulk pseudotyped virus.

Pseudotyped virus titration (Steps 27–38)

When the pseudotyped virus is titrated, one tube of pseudotyped virus is taken out from −80 °C. Pseudotyped virus titration (Fig. 2a) is carried out by using 96-well plates. An initial 10-fold dilution is made followed by threefold serial dilutions for a total of nine dilutions, with each dilution being made in six replicate wells. After dilution, the target cells digested by trypsin are added, and a cell control without pseudotyped virus is also set up. The VSV genome packaged by the pseudotyped virus contains the *Fluc* gene. After the pseudotyped virus infects the target cells, the amount of pseudotyped virus infection can be characterized by adding substrate to detect the content of Fluc expressed. After incubation at 37 °C and 5% (vol/vol) CO_2 for 24 h, the luciferase substrate is added for chemiluminescence detection. The detected raw data are imported into the pseudoviral titration macro TCID50_SARS-CoV-2 (Supplementary Method 1 and Box 1) to calculate the pseudoviral titer and the recommended dilution for the neutralization test (Fig. 3a–c). The 50% tissue culture

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Box 1 | How to use the macros to calculate titers of pseudotyped viruses and EC_{50} for samples

Procedure
1. Export the original data read by the instrument to an .xlsx file (Fig. 3a,d).
2. Open the macro file TCID50_SARS-CoV-2 (Fig. 3b, Supplementary Method 1) or Neutralization_SARS-CoV-2 (Fig. 3e, Supplementary Method 2).
3. Fill in the test information (Fig. 3b,e).
4. Copy the B2–G11 data in the original data .xlsx file. Paste into the original data area (Fig. 3b,e) in the macro file.
5. The titer of pseudotyped virus and the recommended dilution multiple (Fig. 3c) and the EC_{50} value of the sample (Fig. 3f) are calculated automatically by the macros.
   ▲ CRITICAL   The information colored in green in the assay parameter box (Fig. 3c,f) can be modified as needed. If the test sample in the neutralization assay is a monoclonal antibody or entry inhibitor with known concentrations, you can change the 'Dilution Factor' in the assay parameter in macrofile 'Neutralization_SARS-CoV-2' to 1/3 (threefold serial dilution) before calculation. The concentration decreases threefold with the increase of one dilution. The 'Start Dilution' should be changed to the initial concentration of the corresponding sample.

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infectious dose ($TCID_{50}$) of the pseudotyped virus is calculated according to the Reed-Muench method[25] in the titration macro template.

Neutralization assay (Steps 39–48)

The SARS-CoV-2 pseudotyped virus neutralization test begins with serial dilutions of the samples to be tested (Fig. 2b), which are then mixed with a certain amount (325–1,300 $TCID_{50}$/ml) of pseudotyped virus. After that, the target cells are incubated for 24 h, and the amount of pseudotyped virus entering the target cells is calculated by detecting the expression of luciferase, to obtain the neutralizing antibody content of the sample. Following this protocol, four samples (Fig. 2b) can be detected simultaneously in a 96-well plate. The cell control (CC) with only cells and the virus control (VC) with virus and cells are set up in each plate. When the raw data for control and samples are exported from the luminometer and pasted into the macro Neutralization_SARS-CoV-2 (Supplementary Method 2 and Box 1), the half maximal effective concentration (EC_{50}) is calculated for the tested samples (Fig. 3d–f).

Advantages and limitations

Because the S protein of SARS-CoV-2 is considered the main antigen to induce the neutralizing antibody, several immune assays based on full-length S protein[26] or partial regions of S protein such as the RBD region[27,28] have been developed to evaluate the binding antibody for vaccines or in patients; however, it is difficult to predict the protective situations in patients, and the binding antibody may not be comparable between vaccines and patients. The most direct method to evaluate the neutralizing antibodies induced by SARS-CoV-2 vaccine is to use the infection inhibition test with live virus[24], but the handling of live virus must be carried out in BSL-3 laboratories and is limited by experimental conditions and virus accessibility; thus, it is difficult to standardize between laboratories.

Binding assays with full-length or partial S protein detect all the antibodies targeting the coating antigen[26–28]. They do not discriminate between neutralizing antibodies and non-neutralizing antibodies that rarely tend to be protective. The efficacy of the entry inhibitors cannot be evaluated by using the binding assay. Compared to binding assays, the assay based on pseudotyped virus can detect neutralizing antibodies and viral entry inhibitors, which can predict their function and efficacy. Compared to a neutralizing assay with live virus, this assay is safer and can be easily carried out in BSL-2 conditions because the pesudotyped viruses can replicate only for a single cycle after entering cells. Meanwhile, pseudotyped viruses carry reporter genes, which can be expressed after infection to facilitate detection. The quantitative detection of infected virus is realized by recording data objectively with a luminescence meter, which makes the assay amenable to high-throughput detection and easy to standardize.

The SARS-CoV-2 pseudotyped virus neutralization assay can, however, evaluate neutralizing antibodies only for S protein, not for other structural or non-structural proteins. Whether those proteins can induce neutralizing antibody is controversial[29,30]. After the pseudotyped virus infects the cells, it cannot complete the same life cycle as the live virus, and it is impossible to evaluate the inhibitory effect of the product on viral replication. The number of S protein molecules expressed on the surface of the pseudotyped virus may affect the sensitivity of the neutralizing assay. Whether the number of S protein molecules expressed on the surface of the pseudotyped virus is
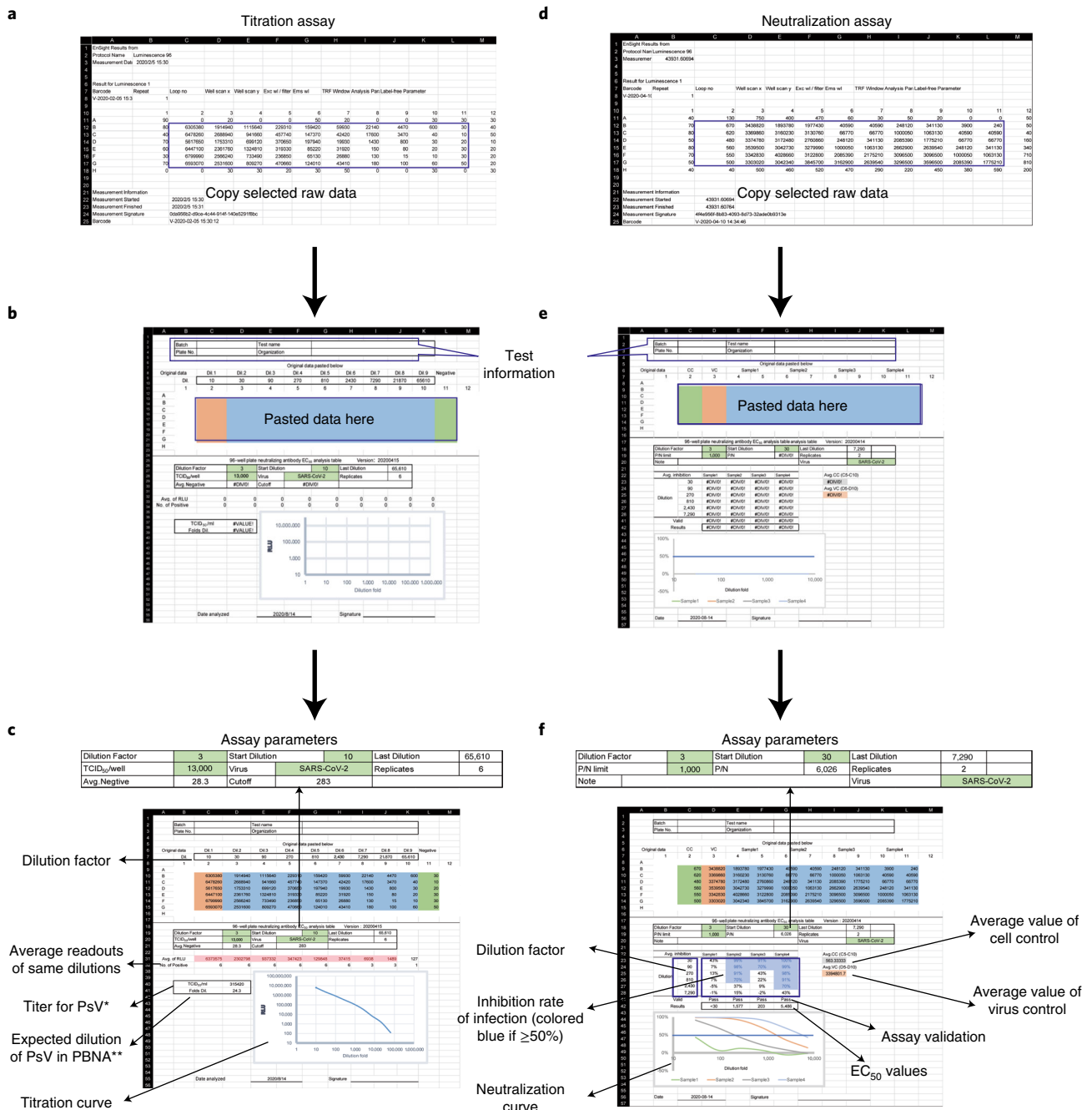
Fig. 3 | Screenshots showing the use of Excel macros to calculate TCID$_{50}$ (Supplementary Method 1 and Box 1) for titration assay and titers for neutralization assay (Supplementary Method 2 and Box 1). a, Luminescence readout data for a titration assay exported in the form of an Excel file. The selected raw data are indicated in a rectangle. **b**, The macro file for titration of the pseudotyped virus, TCID50_SARS-CoV-2 (Supplementary Method 1). A rectangle indicates where the raw data should be pasted. **c**, Calculated results in the TCID50_SARS-CoV-2 macro (Supplementary Method 1). **d**, Luminescence readout data for a neutralization assay exported in the form of an Excel file. The selected raw data are indicated in a rectangle. **e**, The macro file for the neutralization assay, Neutralization_SARS-CoV-2 (Supplementary Method 2). A rectangle indicates where the raw data should be pasted. **f**, Calculated results in the Neutralization_SARS-CoV-2 macro (Supplementary Method 2). Dil., dilution; EC$_{50}$, half maximal effective concentration; P/N, average of virus control/average of cell control; *PsV, pseudotyped virus; **PBNA, pseudotyped virus–based neutralization assay; RLU, relative light unit; TCID50, 50% tissue culture infectious dose; TRF, time-resolved fluorescence.

similar to that of the live virus also needs to be further studied. Therefore, in the process of developing the SARS-CoV-2 pseudotyped virus neutralization assay, it is necessary to carry out comparisons to the live virus to ensure its representativeness to the live virus detection method. Preliminary studies in our laboratory have shown a good correlation between pseudotyped virus

neutralization assay and live virus neutralization assay (J.N., Q.L., J.W., W.H. and Y.W., unpublished observations, 2020).

Applications
The SARS-CoV-2 pseudotyped virus neutralization assay, developed in our laboratory[23], has been used to screen monoclonal neutralizing antibodies[31–33], characterize the neutralizing antibodies in convalescent patients[1,27] and volunteers inoculated with candidate vaccine[34] and evaluate potency of small molecular inhibitors (J.N., Q.L., J.W., W. Wang, W.H. and Y.W., unpublished observations, 2020). By replacing the S protein expression plasmid, the cross-neutralization of antibodies to different mutant strains can be studied[35], and even the cross-neutralization between different coronaviruses can be investigated[31,36]. In addition to being used to evaluate products such as neutralizing antibodies and entry inhibitors in the process of virus infection, the assay can also be used to study virus cell tropism[36] and receptor recognition patterns.

Alternative methods
In addition to the VSV-based pseudotyped virus system, lentivirus-based pseudotyped virus systems are also used in the preparation of SARS-CoV-2 pseudotyped virus[37]. The two systems differ in their backbones; the former is derived from VSV, whereas for the latter, the backbone comes from lentiviruses such as HIV[36] or murine leukemia virus[38]. Compared with VSV-based SARS-CoV-2 pseudotyped viruses, lentivirus-based SARS-CoV-2 pseudotyped viruses have a relatively low pseudoviral titer, for which the relative light unit (RLU) is ~10^6, and can be applied only to hACE2 overexpressed cell lines[36,37]. hACE2 has been identified as the receptor for SARS-CoV-2 to enter host cells[24,39]. It is expected that overexpressed receptors on the host cell surface can enhance the efficiency of infectivity for SARS-CoV-2 pseudotyped virus. The low-titer lentivirus-based SARS-CoV-2 pseudotyped viruses have to employ the hACE2 overexpressed cell lines to yield robust data. The RLU value of SARS-CoV-2 pseudotyped virus based on VSV can reach 10^8, which is 100 times as high as that of the lentivirus system, which means the signal-to-noise ratio of the assay becomes higher and makes the precision and repeatability better. It can be used in cell lines such as Huh-7 or Vero, which are more accessible. In addition, a SARS-CoV-2 neutralization test based on lentivirus-produced pseudovirus will take 48–60 h for incubation after infection, whereas VSV-based pseudotyped virus neutralization assays take just 20–28 h for incubation. VSV replicates in the cytoplasm[40,41]. Conversely, the lentivirus must be reverse-transcribed during replication, which involves transportation of genetic materials between the nucleus and cytoplasm[42,43]. Thus, lentiviral replication takes longer than for VSV. The shorter incubation time may be attributed to the shorter replication time for VSV. This can save time and improve the detection efficiency.

Experimental design
Testing for contamination
To check for potential contamination of the SARS-CoV-2 pseudotyped virus stock with residual G*ΔG-VSV, the SARS-CoV-2 pseudotyped virus stock should be tested by using a VSV G–specific antibody sample when the pseudotyped virus is first used. In our laboratory, the VSV G–specific antibody was generated by immunizing Balb/c mice with VSV G–expressing plasmid at an amount of 20 µg/mouse three times at intervals of 2 weeks. 2 weeks after the final inoculation, blood samples were collected from anesthetized mice by retro-orbital vein bleed, incubated for 1 h at 37 °C and then centrifuged (2,000g at room temperature (22–28 °C) for 10 min) to recover the serum. So far, residual G*ΔG-VSV has not been detected in the SARS-CoV-2 and other pseudotyped virus stocks in our laboratory[23]. Thus, we suggest that this procedure could be omitted if strictly following this protocol.

Validation and controls
The amount of pseudotyped virus added in the neutralization assay will affect the precision and repeatability of the approach. The amount of virus should be kept within a relatively fixed range. According to the optimization results of this assay previously reported, the amount of virus added was 650 $TCID_{50}$ per well (325–1,300 $TCID_{50}$ per well)[23]. To validate the test operation process, the coefficient of variance (CV) of replicates should be controlled within 30% across six wells. The CV of the duplicate wells for samples is also required to be ≤30%. In addition to this CV control of each test plate, we also set up negative (NC) and positive (PC) internal controls for each assay. The NC is human sera collected before the outbreak of COVID-19. The PC serum was from convalescent

patients with COVID-19. The NC and PC samples should be tested along with the testing samples in each run. The EC_{50} value for NC and PC samples used in our laboratory was tested for 30 independent runs. All NC samples were negative at 30-fold dilution. The EC_{50} for PC samples was calculated, and its range of the mean ± 2 s.d. was used as the criterion for the PC. At present, the EC_{50} ranges of quality controls used in our laboratory are <30 for the NC and 978–2,376 for the PC. Because of restrictions on the export of human samples, the internal control cannot be provided to laboratories that want to establish the method, especially foreign laboratories. It is suggested that laboratories planning to establish the SARS-CoV-2 pseudotyped virus neutralization assay should develop their own internal quality controls to monitor the method.

The validity of each sample, each plate and each run is evaluated against a set of pre-specified acceptance criteria. The percent CV (%CV) for duplicate wells checks the validity of individual sample. The %CV of VC wells, and the ratio of VC to CC checks the validity of the plate. The EC_{50} criterion of the NC and the PC validates the test run. The pass/fail criteria used in this assay are listed below:

1   The %CV for duplicate wells is ≤30% for sample dilutions.
2   The %CV of the RLU in the VC wells is ≤30%.
3   The average RLU of VC wells is ≥1,000 times the average RLU of CC wells.
4   The EC_{50} value of the negative sample derived from human serum is <30 (ref. [23]).
5   The EC_{50} value of the positive control is 978–2,376.

If the first criterion is not met, then the result for this sample is deemed invalid. The test for this sample should be re-tested. If the second or third criteria are not met, then the results for this plate are deemed invalid and should be re-tested. If the fourth or fifth criteria are not met, then the results for this run are all deemed invalid and should be re-tested.

Exportation and calculation of results

To calculate the concentration of neutralizing antibody or inhibitor in the tested samples, the RLU values read by the luminometer should be exported as a Microsoft Excel file. Just copy the data of the wells B2–G11 of the exported Excel file and paste them into the corresponding cells in the 'Neutralization_SARS-CoV-2' macro (Supplementary Method 2 and Box 1), and the EC_{50} value of each sample will be calculated automatically. In this procedure, the infection inhibition rates of each dilution of the sample are calculated according to the RLU values as follows: inhibition rate = [1 – (average RLU of sample – average RLU of CC)/(average RLU of VC – average RLU of CC)] × 100%. On the basis of the results of inhibition rate, the EC_{50} of each sample is calculated by the Reed-Muench method. All of these procedures have been incorporated into the 'Neutralization_SARS-CoV-2' macro.

Materials

Biological materials
- Cell line used for pseudotyped virus generation: HEK293T cells (American Type Culture Collection, cat. no. CRL-3216; RRID: CVCL_0063)
- Cell line used for pseudotyped virus infection: Huh-7 cells (JCRB, cat. no. JCRB0403; RRID: CVCL_0336) **! CAUTION** The cell lines should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- pcDNA3.1.S2 recombinant plasmid (GenBank: MT613044), which was constructed by inserting the codon-optimized *S* gene of SARS-CoV-2 (GenBank: MN908947) into pcDNA3.1 plasmid by using BamH I and Xba I restriction sites[23]. It can be shared upon request, or you can get the plasmid through Addgene (Addgene ID: 149457). The plasmid map can be found in Supplementary Fig. 1
- pcDNA3.1.VSVG recombinant plasmid (GenBank: MT613045), which was constructed by inserting codon-optimized VSV *G* gene (Genbank: M27165) into pcDNA3.1. It can be shared upon request, or you can get the plasmid through Addgene (Addgene ID: 158528). The plasmid map can be found in Supplementary Fig. 2
- G*ΔG-VSV, a VSV G pseudotyped virus, in which the *G* gene of the VSV genome was replaced by *Fluc* reporter genes. It was kindly provided by M. A. Whitt[17]. It can be obtained from Kerafast (EH1020-PM; https://www.kerafast.com/productgroup/172/pseudotyped-g-luciferase-gg-luciferase-rvsv)
- *Animal serum samples.* The mice serum samples in this protocol were collected from Balb/c mice (females, ~15 g in weight) inoculated three times with 20 μg of plasmid pcDNA3.1.S2 followed by electroporation at an interval of 2 weeks[22]. 2 weeks after the final inoculation, blood samples were

collected from anesthetized mice by retroorbital vein bleed, incubated for 1 h at 37 °C and then centrifuged (2,000g at room temperature for 10 min) to recover the serum. **! CAUTION** All animal experiments have to be performed in accordance with the ethical guidelines of the institution. All the animal experiments in this protocol were carried out according to the protocols approved by the Institutional Animal Care and Use Committee of the National Institutes for Food and Drug Control.

- *Human serum samples.* The SARS-CoV-2 negative and positive serum samples used in this protocol were kindly provided by Qiang Gao from Sinovac Biotech. The test samples of convalescent sera (CS3 and CS4) were provided by Xiaoming Yang from China National Biotec Group. **! CAUTION** All experiments involving human samples must be approved by the ethics committee. Informed consent has to be obtained from the involved individuals.

Reagents

- Qiagen plasmid plus maxi kit (Qiagen, cat. no. 12963)
- DMEM (high glucose; Hyclone, cat. no. SH30243.01)
- Opti-MEM I reduced serum medium (Gibco, cat. no. 31985088)
- FBS (Pansera ES; PAN-Biotech GmbH, cat. no. ST30-2602)
- PBS (Hyclone, cat. no. SH30256.01)
- Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, cat. no. L3000015) **! CAUTION** This reagent is toxic. Avoid contact, ingestion and inhalation. Wear gloves and handle with care.
- 0.25% trypsin-EDTA (Gibco, cat. no. 25200056)
- HEPES (Genom, cat. no. GNM-11344; or Gibco, cat. no. 15630080)
- Penicillin-streptomycin solution (Genom, cat. no. GNM15140; or Gibco, cat. no. 15140163) **! CAUTION** This reagent is hazardous. Avoid contact, ingestion and inhalation. Wear gloves and handle with care. Those who are allergic to penicillin should pay special attention to it.
- Britelite plus reporter gene assay system (PerkinElmer, cat. no. 6066769)
- DH5α chemically competent cells (Zomanbio, cat. no. ZC101; or Invitrogen, cat. no. 12034013)
- Ampicillin sodium salt (VWR, cat. no. 0339-100G) **! CAUTION** This reagent is hazardous. Avoid contact, ingestion and inhalation. Wear gloves and handle with care. Those who are allergic to penicillin should pay special attention to it.
- Tryptone (Oxoid, cat. no. LP0042)
- Yeast extract (Oxoid, cat. no. LP0021)
- Agar powder (Meitan, cat. no. 11Y03)

Equipment

- Bacterial coating rod (Coolwind, cat. no. QFC01-LS0015D)
- Cell culture tube (BD Falcon, cat. no. 352006)
- Triangle bottle (Nalgene, cat. no. 4112-0250)
- Acrodisc syringe filters with supor membrane (Sterile 0.45 µm, 25 mm; Pall, cat. no. 4614)
- Serological pipettes: 5, 10 and 25 ml (Costar, cat. nos. 4487, 4488 and 4489)
- Cell culture plate (96-well clear polystyrene microplates; Corning, cat. no. 3599)
- Cell culture flask (75 cm²; Corning, cat. no. 430641)
- Centrifuge tubes: 15 and 50 ml (Corning, cat. nos. 430790 and 430828)
- Microtubes: 1.5 and 2 ml (Axygen, cat. no. MCT-150-C and MCT-200-C)
- Chemiluminescence detection plates (96-well; Xiamen Labware, cat. no. 3599; or PerkinElmer, cat. no. 6005290)
- QIAvac24 Plus (Qiagen, model no. 19413)
- NanoDrop One microvolume UV-visible spectrophotometer (Thermo Scientific, model no. ND-ONE)
- Inverted microscope (Zeiss, model no. Primovert)
- Cell counter (Nexcelom, model no. Cellometer Auto T4)
- Cell culture incubator (5% (vol/vol) CO_2 humidified, 37 °C; NuAire, model no. NU-5810E)
- Electroporation instrument (Teresa, model no. Gene Delivery Device A)
- Luminometer (PerkinElmer, model no. HH34000000)

Reagent setup

- *LB medium.* Prepare by adding 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and deionized water to 1 liter. Divide into aliquots and distribute to 250-ml flasks (100 ml per flask). Seal with seal film and autoclave. Store at 2–8 °C for ≤2 weeks before use.

- *Solid LB medium.* Prepare by adding 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 12 g of agar and deionized water to 1 liter. Divide into aliquots and distribute to 1,000-ml flasks (500 ml per flask). Seal with seal film and autoclave. When the temperature of the medium decreases to 70–80 °C, divide into aliquots and distribute to 100-mm cell culture plates (20 ml per plate). When the medium has solidified completely, transfer the plates to 2–8 °C. Store at 2–8 °C for ≤2 weeks.
- *Complete DMEM.* Prepare by adding 10% (vol/vol) FBS, 2% (vol/vol) HEPES and 1% (vol/vol) penicillin–streptomycin to DMEM. Stored at 2–8 °C for ≤7 d before use.
- *Cell wash solution.* Prepare by adding 1 ml of FBS to 99 ml of PBS to make PBS solution with 1% (vol/vol) FBS. Store at 2–8 °C for ≤7 d before use.

Procedure

pcDNA3.1.VSVG and pcDNA3.1.S2 plasmid stock preparation ● Timing 3 d

1   Transfer 1 vial of DH5α competent bacteria (50 µl per vial) into an ice bath box. When the competent bacteria begin to thaw, add 1 µl of plasmid (pcDNA3.1.VSVG or pcDNA3.1.S2) into it and place it back in ice for 30 min.

2   30 min later, put the competent bacteria into a 42 °C water bath for 45–60 s. Then, place them back into the ice box for 2 min. Next, add 500 µl of LB medium into the transformed competent bacteria and place it in a 37 °C shaker at a speed of 120 rpm for 1 h.

3   After incubation in the shaker, add 50 µl of the bacteria onto a bacterial culture plate (solid LB medium with 100 µg/ml ampicillin) and spread it evenly by using a bacterial coating rod. Next, place the plate into a 37 °C incubator overnight (14–16 h).

4   On the next day, pick a single colony of the bacteria by using a sterile 10-µl pipette tip and put it into a cell culture tube with 5 ml of LB medium (containing 100 µg/ml ampicillin). Then, put the tube back into a 37 °C shaker at a speed of 220 rpm overnight (14–16 h).

5   After the overnight incubation, transfer 100 µl of the cultured bacteria into a 250-ml triangle bottle with 100 ml of LB medium (containing 100 µg/ml ampicillin). Then, put it into a 37 °C shaker at a speed of 220 rpm overnight (14–16 h).

6   Collect the bacteria by centrifuging at a speed of 6,000g for 15 min at 4 °C.

7   Discard the supernatant and resuspend the pelleted bacteria by using 8 ml of Buffer P1. Then, add 8 ml of Buffer P2, mix gently four to six times by inverting and incubate at room temperature for 3 min. Next, add 8 ml of Buffer S3 and mix immediately by inverting four to six times. Transfer the solution into a QIAfilter cartridge and incubate at room temperature for 10 min.

8   During incubation, place the spin columns into the QIAvac 24 Plus. Insert tube extenders into each column. Then, push the plunger into the cartridge and filter the cell lysate into 50-ml centrifuge tubes. Next, add 5 ml of Buffer BB and invert it four to six times.

9   Transfer the lysate solution to Qiagen plasmid plus spin columns placed on the QIAvac24 Plus. Apply a vacuum of ~300 mbar until all the solution has been drawn through the columns. Then, switch off the vacuum. It takes ~5–10 min.

10 Add 700 µl of Buffer ETR into the spin column and apply vacuum until the filter is dried.

11 Place the spin columns into 2-ml tubes and centrifuge at 10,000g for 1 min at room temperature to remove the residual buffer.

12 Place the spin columns into clean 2-ml tubes, add 400 µl of Buffer EB to the spin columns and let them stand for 2 min. Then, centrifuge for 1 min to collect the purified plasmids.

13 Determine the concentration of plasmid by using a spectrophotometer. If $OD_{260}/OD_{280} = 1.8$–2.0, $A_{260} > 0.1$ and $A_{320} \leq 0.01$, then the plasmid meets the quality criteria for transfection.

14 Make 50-µg aliquots of the plasmid in a sterile 2-ml tube and store at −80 °C until use.
   ■ **PAUSE POINT** The plasmids can be stored at −80 °C for ≤1 year before use.

G*ΔG-VSV and SARS-CoV-2 pseudotyped virus stock preparation ● Timing 3 d

▲ CRITICAL G*ΔG-VSV pseudotyped virus is needed for producing SARS-CoV-2 pseudotyped virus. G*ΔG-VSV can initially be obtained from Kerafast (EH1020-PM). However, it is only an aliquot with 300 µl of working stock, which is not enough to generate the SARS-CoV-2 pseudotyped virus. Thus, it is necessary to generate additional working stocks by amplifying G*ΔG-VSV by using the steps in this

section (and plasmid pcDNA3.1.VSVG in Step 18) before production of SARS-CoV-2 pseudotyped virus, also following this section (and using plasmid pcDNA3.1.S2 in Step 18).

Cell preparation for pseudotyped virus production

15 We maintain the 293T cells in complete DMEM medium and prepare cells for pseudotyped virus production as described below. First, when the 293T cells grow to a 70–90% confluence in a T75 flask, carefully discard the supernatant culture medium of the flask. Then, add 10 ml of 37 °C pre-warmed PBS to the flask to clean the cell surface. Next, discard the cleaning solution and add 3 ml of 0.25% trypsin-EDTA to the flask to cover the cells. Rest at room temperature for 1 min and discard trypsin by pipetting. Transfer the cells to a 37 °C, 5% (vol/vol) CO_2 incubator and incubate for 5 min. After that, add 10 ml of DMEM complete medium preheated at 37 °C to the cell culture flask and resuspend the cells thoroughly by repeated pipetting with a serological pipette to make sure the cells are dispersed into a single cell suspension.
   ? TROUBLESHOOTING
16 Count the cells by using a cell counter and adjust the cell concentration to $5–7 \times 10^5$ cells/ml with DMEM complete medium. Then, transfer 15 ml into a T75 cell flask and place it in a 5% (vol/vol) CO_2 cell incubator at 37 °C overnight.

Transfection and infection

17 Check the cell density before cell transfection. The cell density should reach 70–90% confluence after overnight incubation.
   ▲ CRITICAL STEP If the cell density has not reached the expected confluence, replace the culture medium and lengthen the incubation time for another 4–6 h.
18 Add 0.75 ml of opti-MEM into a new 2-ml microtube (labeled 'tube A'). Then, add 30 µg of plasmid pcDNA3.1.VSVG (or plasmid pcDNA3.1.S2) into tube A. Next, add 30 µl of P3000 (a component in the Lipofectamine 3000 kit) into tube A and mix it by using a pipette. Rest it at room temperature for 5 min.
19 Transfer 0.75 ml of opti-MEM into another 2-ml microtube (marked as 'tube B') and add 30 µl of Lipofectamine 3000 into it. Mix the solution gently and place it at room temperature for 5 min.
20 After a 5-min incubation, add the solution of tube B to tube A drop by drop. Invert tube A four to six times gently to mix the mixture completely, and rest it at room temperature for 15–20 min.
   ▲ CRITICAL STEP Make sure that the mixing is gentle and complete, which will influence the efficiency of transfection.
21 During the room temperature incubation (Step 20), dilute G*ΔG-VSV (VSV G pseudotyped virus) to 7.0×10^4 $TCID_{50}$/ml. Discard the culture medium of 293T cells from Step 17 and then add 15 ml of the diluted G*ΔG-VSV solution into the T75 flask.
22 After the incubation in Step 20 comes to an end, pipette all the solution in tube A into the supernatant of the 293T cell flask and mix it by slowly pipetting four to six times. Place it in a 5% (vol/vol) CO_2 incubator at 37 °C for 6–8 h or overnight.
23 After the incubation, aspirate the culture medium and wash the cells by using 10–15 ml of cell wash solution (PBS containing 1% (vol/vol) FBS) to rinse the cells gently and thoroughly. Repeat this step three times.
   ▲ CRITICAL STEP This wash step should be performed carefully and gently to avoid detaching the cells. Detached cells will yield lower-titer or even no pseudotyped viruses.
   ? TROUBLESHOOTING
24 Add 15 ml of fresh complete DMEM to the flask and place it in a 5% (vol/vol) CO_2 incubator at 37 °C for 24 h.
25 Collect the cell supernatant (supernatant A) into a 50-ml centrifuge tube by pipetting and place it in a 4 °C refrigerator. Then, add 15 ml of fresh complete DMEM into the 293T cells and incubate for another 24 h.
26 After the second incubation, harvest the supernatant (supernatant B) and mix it with supernatant A by pipetting. Centrifuge the mixture at 1,000*g* for 10 min at room temperature. Filter by using a 0.45-µm filter. Distribute 1-ml aliquots of the mixture into 2-ml microtubes and store them at −80 °C.
   ■ PAUSE POINT The harvested pseudotyped virus can be stored at −80 °C for ≤1 year according to the conditions used for other pseudotyped viruses[17,22].

Titration of the SARS-CoV-2 pseudotyped virus ● Timing 2d

27 Take the pseudotyped virus from the −80 °C refrigerator and thaw it in a room temperature water bath.

28 Use a 96-well flat-bottom culture plate to titrate the pseudotyped virus. Seal the outmost wells with 250 μl of sterilized water per well to avoid the effect caused by evaporation of the edge-well culture medium (Fig. 2a).

29 Place 135 μl of complete DMEM in wells B2–G2. Add 100 μl of complete DMEM into the remaining wells (B3–G11). Add 15 μl of pseudotyped virus per well to wells B2–G2 to make an initial dilution of 1:10.

30 Start the serial dilution by transferring 50 μl of solution from wells B2–G2 to wells B3–G3 with a multi-channel pipette, mix six to eight times and then transfer 50 μl from wells B3–G3 to wells B4–G4. Repeat the transfer and dilution of samples through to wells B10–G10. After the final mixing, aspirate 50 μl of solution from each well in column 10 and discard it. The dilution step does not involve column 11 (B11–G11), which will be used as the CC.

31 Prepare Huh-7 cells as described in Step 15. Then, adjust the cell concentration to 2×10^5 cells/ml with DMEM complete medium and add 100 μl of cell suspension into all of the 60 inner wells (B2–G11).

32 Place the titration plate in the 5% (vol/vol) CO_2, 37 °C incubator for 20–28 h.

33 After the incubation, thaw the required volume of the Britelite plus reagent before use in an ambient temperature water bath.
▲ CRITICAL STEP The temperature of Britelite plus reagent can influence the readout of the sample. Place the reagent in a water bath not less than 30 min before use.

34 Remove and discard 100 μl of supernatant from each well, leaving ~100 μl of liquid in each well.

35 Disperse 100 μl of the Britelite plus reagent into each well.

36 Incubate in darkness at room temperature for 2 min to allow complete cell lysis. Mix by pipettor action (six to eight times) and transfer 150 μl to a corresponding 96-well chemiluminescence detection plate.
▲ CRITICAL STEP Note that the transparent cell culture plate is not suitable for chemiluminescence detection. The cell lysates should be transferred to specific plates for detection.

37 Read the RLU of the plate after the 2-min incubation in the luminescence meter.

38 Take 10 times of the average RLU of the CC wells (B11–G11) as the cutoff value. Wells with an RLU equal to or higher than the cutoff value are considered positive. On the basis of the positive and negative rate in each dilution, calculate the $TCID_{50}$ according to the Reed-Muench method by using the 'TCID50_SARS-CoV-2' macro (Supplementary Method 1 and Box 1). Figure 3 shows how to use the macro file to calculate the $TCID_{50}$ for pseudotyped virus titration.
? TROUBLESHOOTING

Pseudotyped virus neutralization assay ● Timing 1 d

39 *Sample preparation*. Serum samples from animals or humans should be inactivated in a water bath at 56 °C for 30 min.
! CAUTION All animal experiments have to be carried out in accordance with the ethical guidelines of the institution. All experiments involving human specimens should be approved by the ethics committee. Informed consent must be obtained from the involved individuals.
? TROUBLESHOOTING

40 Prepare a culture plate and seal the outmost wells as in Step 28.

41 Place 150 μl of complete DMEM medium per wellin column 2 (B2–G2), which is used as the CC (Fig. 2b). Add 100 μl of complete DMEM into each well of column 3 (B3–G3), which is designated as the VC.

42 Dispense 142.5 μl of complete DMEM per well in the remaining wells of row B (B4–B11), which is the initial dilution row. Add 100 μl of complete DMEM per well to the remaining wells (C4–G11).

43 Add 7.5 μl of the test sample in duplicate to row B (B4–B11) in the following order: sample 1, wells B4–B5; sample 2, wells B6–B7; sample 3, wells B8–B9; sample 4, wells B10–B11.
▲ CRITICAL STEP After addition of the sample, rinse the tips by using the medium in the corresponding wells to avoid leaving residual samples on the tip walls. 7.5 μl of sample + 142.5 μl of medium yields a sample dilution of 1:20.
▲ CRITICAL STEP A positive and a negative control should be included in at least one plate in each run of the assay. The positive and negative controls are treated the same as the test samples.

44 Mix the samples in row B by pipetting with a multi-channel pipette and transfer 50 μl from each well in row B to row C. Repeat the mixing and transfer through row G (they are threefold serial

dilutions). After final transfer and mixing, discard 50 µl of liquid from the wells in the final row of columns 4–11 (wells G4–G11).

45 Thaw the required amount of pseudotyped virus in an ambient temperature bath. Dilute the SARS-CoV-2 pseudotyped virus to 1.3×10^4 TCID$_{50}$/ml in complete DMEM. Dispense 50 µl of the diluted pseudotyped virus into each well in columns 3–11. Then, cover the plates and incubate in a 5% (vol/vol) CO$_2$, 37 °C incubator for ~60 min (45–90 min).

▲CRITICAL STEP Note that no pseudotyped viruses are added in the CC wells. Pseudotyped viruses are dispensed into VC, negative control and positive control wells in the same way as the test sample wells.

▲CRITICAL STEP After addition of the pseudotyped virus, the sample is diluted another 1.5-fold, which makes the initial dilution 1:30 followed by five serial threefold dilutions. The final dilution of the sample will be 1:7,290.

46 After a 30-min incubation, start to prepare the Huh-7 cells as in Step 31 and when the incubation comes to an end, add cells as in Step 31 and place the titration plate in the 5% (vol/vol) CO$_2$, 37 °C incubator for 20–28 h.

47 After the incubation, repeat Steps 33–37 (removing 150 µl of supernatant from each well in Step 34).

48 Export the raw data as an Excel file from the luminometer and paste the raw data into the Excel 'Neutralization_SARS-CoV-2' macro (Supplementary Method 2 and Box 1) to get the titer for each sample.

▲CRITICAL STEP Figure 3 shows how to use the macro file to calculate the neutralization titers for serum, antibody or fusion inhibitors by changing the wells of dilution factor and start dilution.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
15	The cells are lumpy after plating	Incomplete digestion	Before washing the cells by using PBS, the culture medium should be discarded completely. After cleaning with PBS, aspirate and discard the PBS completely. After discarding trypsin, the cell culture bottle should be placed in the incubator without disturbing the cells
		Over-digestion	Incubate strictly according to the protocol timeline to avoid a prolonged digestion period
23	Cells are detached from the surface of the culture flasks	When washing or adding culture medium, the liquid is added directly to the cells	When rinsing cells or adding culture medium, tilt the culture bottle to make the cellular side face up. Add the liquid to the acellular side. When the liquid flows to the bottom of the flask, turn it gently to make the liquid infiltrate the cell surface. This method should be applied to all cell-processing operations
38	The titers of pseudotyped virus are too low	The transfection efficiency was low	Lipofectamine 3000 should be fully mixed with the plasmid in Step 19. The liquids in tubes A and B should be fully mixed in Step 20 so that the liposome can encapsulate the plasmid efficiently
39	The sample is not clear	The serum/plasma sample contains blood lipids or fiber precipitation	After inactivation, serum or plasma samples should be centrifuged at 6,000g for 10 min. Then, the supernatant will be used for follow-up detection
48	The RLUs for the VC are not exactly the same in different runs	Even diluted according to the same TCID$_{50}$, the RLU may not be exactly the same	Following the recommended dilution fold in the TCID$_{50}$ macro, it is acceptable as long as the VC/CC value is not <1,000 in the pseudotyped virus neutralization assay
	%CV between duplicates is >30%	Inaccuracy of sample addition or dilution	When adding or diluting samples, observe the liquid level in the pipette tips to ensure an identical volume. It is recommended to use a multi-channel pipette to do the serial dilutions

Timing

Step 1, plasmid addition for transformation: 3 min, incubation in ice: 30 min

Step 2, heat shock for transformation: 10 min, incubation in shaker: 1 h

Step 3, bacterial spreading: 5 min, overnight incubation: 14–16 h

Step 4, mono-colony picking: 5 min, overnight incubation: 14–16 h

Step 5, amplified culture of bacteria: 5 min, overnight incubation: 14–16 h

Step 6, bacteria collection: 25 min

Step 7, cell lysis and precipitation: 30 min

Step 8, preparation of the spin column: 15 min

Steps 9–12, washing and collecting the plasmid: 40 min

Step 13, quantifying and determining the quality of the plasmid: 10 min

Step 14, preparing aliquots of the plasmid: 15 min

Steps 15 and 16, cell preparation for pseudotyped virus production: 45 min, overnight incubation: 14–16 h

Steps 17–22, transfection and infection of 293T cells: 1 h, incubation: 6–8 h

Steps 23 and 24, replacement of the transfection and infection solution with culture medium: 30 min, incubation: 24 h

Step 25, first harvest of the pseudotyped virus: 10 min, incubation: 24 h

Step 26, second harvest and aliquoting of the pseudotyped virus: 1 h

Step 27, preparation of the pseudotyped virus for titration: 5 min

Step 28, preparation of the culture plate for titration: 10 min

Steps 29 and 30, pseudotyped virus dilution: 10 min

Steps 31–33, preparation of cells for titration: 45 min, incubation: 20–28 h

Steps 34–37, luminescence detection: 30 min

Step 38, titration calculation by using a macro: 5 min

Step 39, sample preparation: 1 h 20 min

Step 40, preparation of the culture plate for neutralization: 10 min

Steps 41–44, serum or antibody sample dilution: 30 min

Step 45, pseudotyped virus preparation and addition: 10 min, incubation: 1 h

Step 46, preparation and dispensing of cells: 45 min, incubation: 20–28 h

Step 47, luminescence detection: 30 min

Step 48, calculation of the EC_{50} by using a macro: 5 min

Anticipated results

Following this protocol, high-titer SARS-CoV-2S pseudotyped viruses can be obtained. Even if diluted 10 times, the ratio of VC/CC can reach >1,000. The high signal-to-noise ratio could ensure the precision of the assay. The titer of the pseudotyped virus and the expected dilution in the neutralization test can be calculated by using the Excel 'TCID50_SARS-CoV-2' macro (Supplementary Method 1, Box 1 and Fig. 4a). If the protocol is carried out appropriately to detect neutralizing antibodies in serum samples, a well-fitted inhibition curve can be observed automatically in the 'Neutralization_SARS-CoV-2' macro after importing the raw data (Fig. 4b). Four samples could be assayed in duplicate in each 96-well plate at the same time. The neutralizing antibody titer of the sample can be calculated by using the Excel 'Neutralization_SARS-CoV-2' macro (Supplementary Method 2 and Box 1). That is the dilution of a serum sample that inhibits 50% of viral infection. If the test sample is a monoclonal antibody or a fusion inhibitor with an assigned amount, the EC_{50} of the sample can be obtained (Fig. 4b).

Reporting Summary

Further information on the research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

A sample dataset has been deposited in Figshare: https://figshare.com/articles/Raw_data_for_Supplementary_methods_and_Figure_3_and_4/12486479/1. This dataset was used to generate Figs. 3 and 4 and is included as Source Data. Source data are provided with this paper.
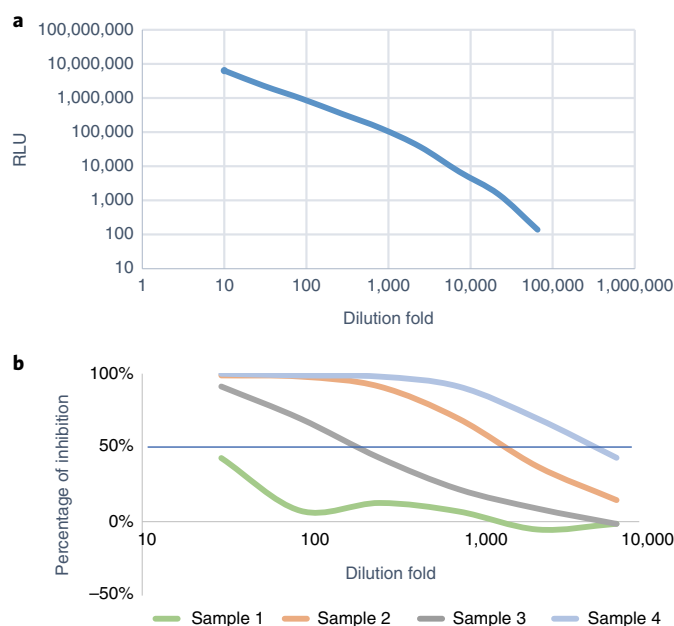
Fig. 4 | Titration (a) and neutralization (b) curves obtained by using the macro files (Supplementary Methods 1 and 2 and Box 1). a, Titration curve generated by using the TCID50_SARS-CoV-2 macro (Supplementary Method 1). The x-axis shows the dilution fold of pseudotyped viruses. The y-axis shows the RLU value of the luminescence meter readout. **b**, Neutralization curve generated by using the Neutralization_SARS-CoV-2 macro (Supplementary Method 2). Four samples can be assayed in the same 96-well plate. A four-parameter curve (S-shaped curve) can be obtained between the inhibition rate and log-transformed dilution fold, when the test sample can inhibit the infection of target cells by pseudotyped virus. It may be a partial S-shaped curve. Samples with low (sample 1), middle (sample 3), or high (samples 2 and 4) neutralization potency present posterior, middle or anterior segments of S curves, respectively, in this figure. When the neutralization curve crosses the 50% line, the EC_{50} for this sample can be calculated. The EC_{50} (thin blue line showing 50% inhibition) was calculated by using the Reed-Muench method automatically in the macro. The raw data have been deposited in Figshare (https://figshare.com/articles/Raw_data_for_Supplementary_methods_and_Figure_3_and_4/12486479/1) and are available with this article as Source Data.

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Acknowledgements

Related links
Key references using this protocol
Nie, J. et al. *Emerg. Microbes Infect.* **9**, 680–686 (2020): https://doi.org/10.1080/22221751.2020.1743767
Li, Q. et al. *Cell* (2020): https://doi.org/10.1016/j.cell.2020.07.012
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