Two Detailed Plaque Assay Protocols for the Quantification of Infectious SARS-CoV-2

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Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been identified as the causal agent of COronaVIrus Disease-19 (COVID-19), an atypical pneumonia-like syndrome that emerged in December 2019. While SARS-CoV-2 titers can be measured by detection of viral nucleic acid, this method is unable to quantitate infectious virions. Measurement of infectious SARS-CoV-2 can be achieved by tissue culture infectious dose−50 ($TCID_{50}$), which detects the presence or absence of cytopathic effect in cells infected with serial dilutions of a virus specimen. However, this method only provides a qualitative infectious virus titer. Plaque assays are a quantitative method of measuring infectious SARS-CoV-2 by quantifying the plaques formed in cell culture upon infection with serial dilutions of a virus specimen. As such, plaque assays remain the gold standard in quantifying concentrations of replication-competent lytic virions. Here, we describe two detailed plaque assay protocols to quantify infectious SARS-CoV-2 using different overlay and staining methods. Both methods have several advantages and disadvantages, which can be considered when choosing the procedure best suited for each laboratory. These assays can be used for several research purposes, including titration of virus stocks produced from infected cell supernatant and, with further optimization, quantification of SARS-CoV-2 in specimens collected from infected animals.

Basic Protocol: SARS-CoV-2 plaque assay using a solid double overlay method
Alternate Protocol: SARS-CoV-2 plaque assay using a liquid overlay and fixation-staining method

Keywords: SARS-CoV-2 • COVID-19 • COVID19 • virus quantification • plaque assay

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INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), formerly known as 2019-nCoV, was identified as the causal agent of COronaVIrus Disease-19 (COVID-19),

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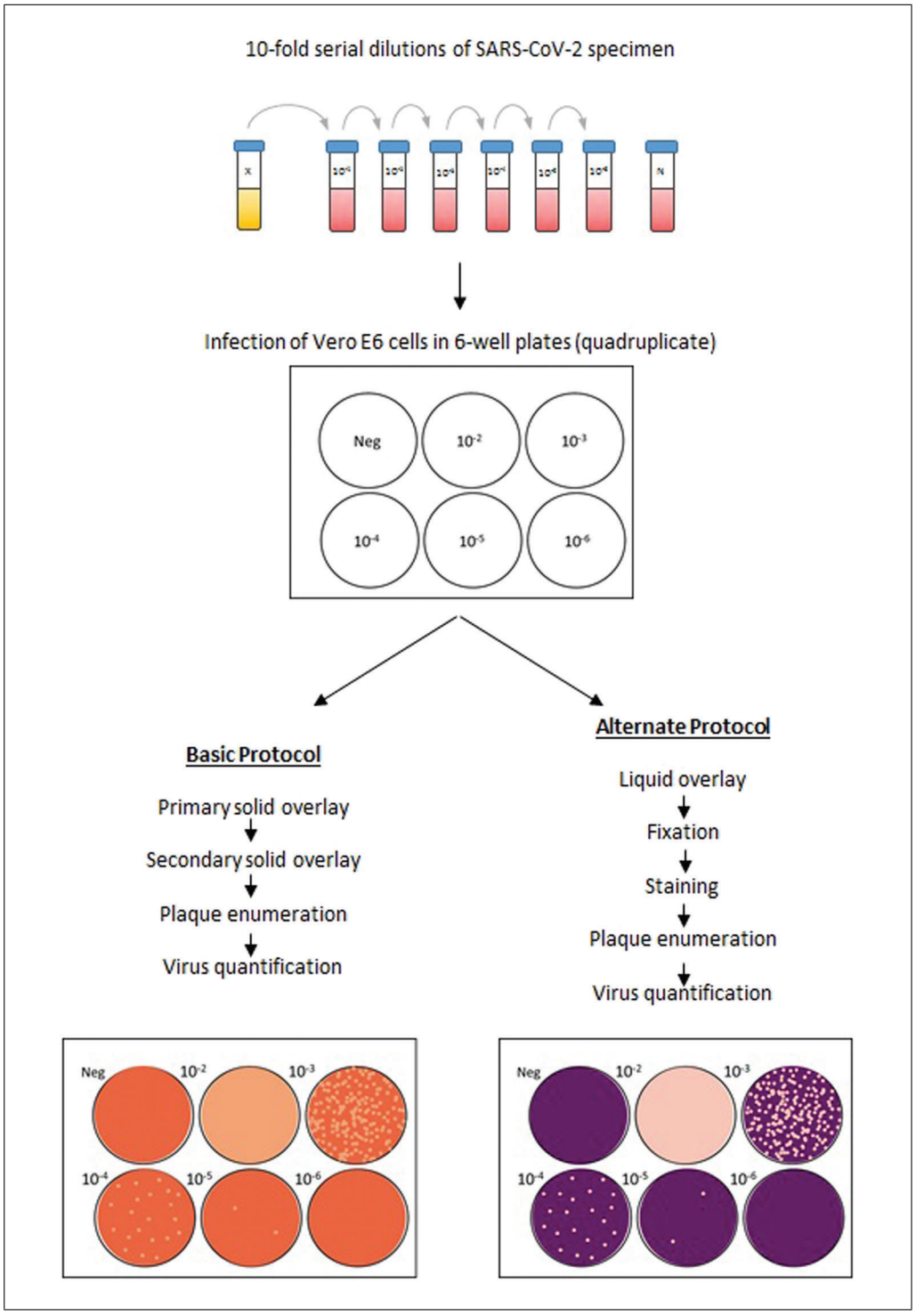

Figure 1 Graphical protocol overview of two plaque assay methods for the quantification of infectious SARS-CoV-2.

a pneumonia-like syndrome that emerged in Wuhan, Hubei, China in December 2019 (WHO, 2020c; Wu et al., 2020; Zhu et al., 2020). Since then, SARS-CoV-2 has spread rapidly, and the pandemic was deemed a public health emergency of international concern in January 2020 (WHO, 2020d). As of May 1, 2020, over 3 million cases and 216,000 fatalities have been reported worldwide (WHO, 2020a). To date, there remains no therapeutic or vaccine effective for the treatment or prevention of SARS-CoV-2 infection (Sanders, Monogue, Jodlowski, & Cutrell, 2020). Thus, clinical management of critically ill patients with COVID-19 relies heavily upon supportive

measures, such as mechanical ventilation and hemodynamic support (Poston, Patel, & Davis, 2020). The enormous number of patients and limited resources have overwhelmed health care systems in their efforts to provide such support. Ongoing research involving infectious SARS-CoV-2 will be crucial in understanding viral-associated pathogenesis and conducting pre-clinical testing of medical countermeasures for the pathogen causing the COVID-19 pandemic.

Virus quantification assays are key tools for research involving SARS-CoV-2. Molecular tests detecting viral RNA can be used to quantify viral loads in clinical samples and virus titers of SARS-CoV-2 stocks prepared from cell culture supernatants (Chu et al., 2020; Corman et al., 2020). While this method is highly sensitive and specific, it is incapable of quantifying infectious SARS-CoV-2, particularly when a high proportion of non-infectious virions are produced during a lytic cycle. Tissue culture infectious dose−50 ($TCID_{50}$) measures infectious SARS-CoV-2 by detecting the presence or absence of cytopathic effect in cell culture upon infection with serial dilutions of a virus specimen (van Doremalen et al., 2020). However, this only provides a qualitative measurement of infectious virus in $TCID_{50}$ units, which describe the amount of virus needed to induce 50% CPE in susceptible cells. Plaque assays are a quantitative method of measuring infectious SARS-CoV-2 by quantifying the plaques formed in cell culture upon infection with serial dilutions of a virus specimen (Harcourt et al., 2020). Infectious virus titers are measured in plaque-forming units (PFU). As such, plaque assays remain the gold standard in quantifying concentrations of replication-competent lytic virions (Cooper, 1961; Juarez, Long, Aguilar, Kochel, & Halsey, 2013).

In this article, we describe two detailed procedures to conduct SARS-CoV-2 plaque assays. In both plaque assays, a confluent monolayer of host cells is infected with serial dilutions of SARS-CoV-2 of unknown starting concentration. After adsorption, an immobilizing overlay is used to cover the infected monolayer, to prevent virus spread and restrict virus growth to foci of cells at the sites of initial infection. During incubation, zones of cell death develop as viral infection and replication are restricted to the surrounding monolayer, leading to plaque formation. After incubation, cells are stained to enhance the contrast between plaques and the uninfected monolayer. Plaques are then enumerated and used to calculate the titer of infectious virus in the specimen. The first plaque assay method that we describe (Basic Protocol) uses Noble agar as the matrix in a solid overlay and neutral red as the stain to enhance plaque visualization. The second plaque assay method that we describe (Alternate Protocol) uses carboxymethylcellulose (CMC) as the matrix in a liquid overlay and crystal violet as the stain to enhance plaque visualization. An overview of these protocols is shown in Figure 1. We believe that both of these protocols would be useful for quantifying infectious SARS-CoV-2 in viral stocks produced from infected cell supernatant. We also believe that either method can be further optimized for other research purposes, such as quantifying infectious SARS-CoV-2 in specimens from animals experimentally infected with SARS-CoV-2.

SARS-CoV-2 PLAQUE ASSAY USING A SOLID DOUBLE OVERLAY METHOD

BASIC PROTOCOL

This protocol outlines a plaque assay method that can be used for the quantification of SARS-CoV-2 plaque-forming units (PFUs) in virus specimens, including viral stocks prepared from infected cell culture supernatants, and with further optimization, bodily fluids from animals infected with SARS-CoV-2. In brief, 10-fold serial dilutions of specimens containing an unknown amount of SARS-CoV-2 are adsorbed onto a monolayer of susceptible cells. After adsorption, a solid overlay medium (S-OM) is applied to the cell monolayer to restrict virus growth to the originally infected foci of cells. These foci develop into plaques, which are visualized after the addition of a secondary S-OM

containing neutral red, which is incorporated and bound by lysosomes in viable cells (Repetto, del Peso, & Zurita, 2008). As a result, clear plaques can be distinguished from the brownish-red uninfected monolayer. Plaques are enumerated and used for the calculation of virus titers in PFU/ml.

Materials

Vero E6 cells (ATCC® CRL-1586™)
Cell maintenance medium (see recipe)
Gibco™ Trypsin-EDTA (0.25%), with phenol red (ThermoFisher Scientific #25200072)
Infection medium (see recipe)
Specimens to be assayed for virus
Gibco™ DPBS, no calcium, no magnesium (ThermoFisher Scientific #14190144)
Overlay diluent (see recipe)
2% Noble agar (NA; solid matrix, see recipe)
0.33% neutral red (NR; see recipe)
Micro-Chem Plus Disinfectant Detergent (National Chemical Laboratories #C849T34)

Class II biological safety cabinet
150-cm^2 (T-150) culture flasks (Corning™ #430825 or equivalent)
CO_2 incubator (Heracell™ 150i CO_2 incubator with stainless-steel chamber, or equivalent)
Light microscope
Falcon 50-ml conical centrifuge tubes (Corning™ #C352098 or equivalent)
Countess II automated cell counter (or equivalent)
Costar® 6-well plates (Corning™ #3506 or equivalent)
2-ml microcentrifuge tubes (ThermoFisher Scientific #50809242PK or equivalent)
Vacuum source
Serological pipettes, sterile:
  5-ml (Corning™ #C4487 or equivalent)
  10-ml (Corning™ #C4488 or equivalent)
  25-ml (Corning™ #C4489 or equivalent)
  50-ml (Corning™ #C4490 or equivalent)
Pipet-Aid (or equivalent)
Repeat pipettor (e.g., Rainin, or equivalent from ThermoFisher Scientific)
Tips for repeat pipettor:
  20-µl barrier tips (ThermoFisher Scientific #2149 or equivalent)
  200-µl barrier tips (ThermoFisher Scientific #2770 or equivalent)
  1000-µl barrier tips (ThermoFisher Scientific #2380 or equivalent)
44°C water bath
Timer
Microwave oven
VWR Scientific TW-26 White Light Transilluminator (or equivalent)

Additional reagents and equipment for basic cell culture techniques including trypsinization (see Current Protocols article: Phelan & May, 2015)

Cell culture (Day 0)

1. *Cell maintenance:* Maintain Vero E6 cells in T-150 flasks containing cell maintenance medium in an incubator at 37°C with 5% CO_2. See Current Protocols article Phelan and May (2015) for basic cell culture techniques.

   Vero CCL-81 cells are also susceptible to SARS-CoV-2 infection (Harcourt et al., 2020). However, it may be necessary to optimize conditions of the protocol, such as infection medium, overlay diluent, and the number of days post-infection to enumerate plaques.

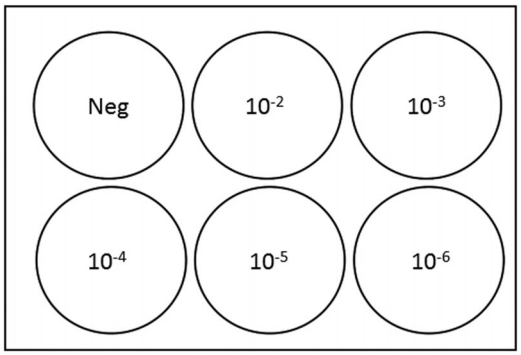

Figure 2 Plate layout for plaque assays conducted in 6-well plates. Vero E6 cells grown in 6-well plates are inoculated with 100 μl of 10-fold serial dilutions of SARS-CoV-2 specimen from 10^{-2} to 10^{-6}, as well as infection medium as a negative control. Four replicates are prepared for each titrated specimen.

2. *Seed plates:* Trypsinize (see Current Protocols article: Phelan & May, 2015) a confluent T-150 flask of Vero E6 cells and bring volume to 20 ml with cell maintenance medium. Use an automated cell counter to count cells and prepare a suspension at a density of 3×10^5 cells/ml in cell maintenance medium. For each specimen to be titrated, seed four 6-well plates with 6×10^5 cells per well by adding 2 ml of the suspension to each well. Incubate at 37°C in a 5% CO_2 incubator overnight to achieve 100% confluence the following day.

   Visualize cells the following day using a light microscope. Proceed with infection if cells have reached 95% to 100% confluence.

Specimen dilution, infection of cells, and primary overlay (Day 1, 0 days post-infection, dpi)

3. *Specimen dilution:* For each specimen to be titrated, label 2-ml microcentrifuge tubes as follows: Neg, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Add 900 μl of infection medium to each tube. Serially dilute specimens 10-fold by transferring 100 μl of each specimen to the appropriate tube labeled 10^{-2}, vortexing, and transferring 100 μl to the subsequent tube, down to 10^{-6}.

   Working with SARS-CoV-2 requires Biosafety Level (BSL) 3−compliant facilities. Work with your institution and safety officers to determine relevant personal protective equipment (PPE) and safety measures for working with infectious virus stocks. See Critical Parameters and also see Current Protocols article Burnett, Lunn, & Coico (2009) for more information.

   For high-titer specimens, it may be necessary to conduct further serial dilutions.

4. *Infection and adsorption:* Label 6-well plates containing cells (see step 2) according to Figure 2. Aspirate medium from cell monolayers under vacuum, leaving ∼100 μl of medium to prevent monolayer from drying. Using a Pipet-Aid and a serological pipette, wash cells with 1 ml of DPBS. Leave ∼100 μl DPBS to prevent monolayer from drying out. Dispense 100 μl of the infection medium from the microcentrifuge tube labeled "N" into designated wells, as depicted in Figure 1. Dispense 100 μl of diluted specimens (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) into designated wells, changing pipette tips every time (Figure 2). Incubate at 37°C in a 5% CO_2 incubator for 1 hr, rocking every 15 min.

   Do not swirl the inoculum. Use a front-to-back and side-to-side movement to evenly distribute the inoculum across the monolayer.

5. *Preparation of primary S-OM:* Incubate overlay diluent at 44°C in a water bath. Microwave to dissolve 2% NA (solid matrix) and incubate at 44°C in a water bath. After rocking plates for the last time (~10 min before incubation is over), prepare primary S-OM by combining equal amounts (1:1) of overlay diluent and 2% NA (solid matrix). Use **Formula 1** to calculate the total volume of S-OM needed, followed by **Formula 2** to calculate the overlay diluent and 2% NA (solid matrix) needed to prepare the primary S-OM.

   Formula 1: Total volume of primary S-OM needed (in ml) = Number of specimens × 4 plates per specimen × 6 wells per plate × 3 ml primary S-OM per well × 1.2 (to account for pipetting error)

   Formula 2: Volume of overlay diluent or 2% NA (solid matrix) needed for primary S-OM (in ml) = total volume of primary S-OM needed ÷ 2

   The bottle of prepared primary S-OM can be placed on top of a Styrofoam mat to prevent it from cooling in the biological safety cabinet (BSC).

6. *Primary solid overlay:* Add 3 ml of primary S-OM to each well of the titration plates (step 4). Allow primary S-OM to solidify and incubate at 37°C in a 5% CO_2 incubator for 2 days.

Secondary overlay and staining (Day 3, 2 dpi)

7. *Preparation:* Incubate overlay diluent at 44°C in a water bath. Microwave to dissolve 2% NA (solid matrix) and incubate at 44°C in a water bath. Prepare secondary S-OM (composition: 1:1 overlay diluent:2% NA with 0.01% neutral red) by using **Formula 3** to calculate the total volume of secondary S-OM needed. Use **Formula 4** to calculate the volume of 0.33% NR needed, **Formula 5** to calculate the volume of overlay diluent needed, and **Formula 6** to calculate the volume of 2% NA (solid matrix) needed to prepare the secondary S-OM.

   Formula 3: Total volume of secondary S-OM needed (in ml) = Number of specimens × 4 plates per specimen × 6 wells per plate × 2 ml secondary S-OM per well × 1.2 (to account for pipetting error)

   Formula 4: Volume of 0.33% NR needed for secondary S-OM (in ml) = total volume of secondary S-OM needed × 0.03

   Formula 5: Volume of overlay diluent needed for secondary S-OM (in ml) = (total volume of secondary solid overlay medium needed ÷ 2) − volume of 0.33% NR needed for secondary S-OM

   Formula 6: Volume of 2% NA (solid matrix) needed for secondary S-OM = total volume of secondary S-OM needed ÷ 2

   The bottle of prepared secondary S-OM can be placed on top of a Styrofoam mat to minimize cooling in the BSC.

8. *Secondary solid overlay:* Add 2 ml of secondary S-OM to each well of the titration plates. Allow secondary S-OM to solidify and incubate at 37°C in a 5% CO_2 incubator overnight.

Enumeration of plaques and titer calculation (Day 4, 3 dpi)

9. *Enumerate plaques:* Use a white-light transilluminator (light box) to aid in visualizing the plaques, which will appear as peach-colored circles on a brownish-red monolayer of cells (Figure 3A). The negative control should have a uniform monolayer, which can be used as a reference. Record the number of plaques observed per well at each virus dilution.

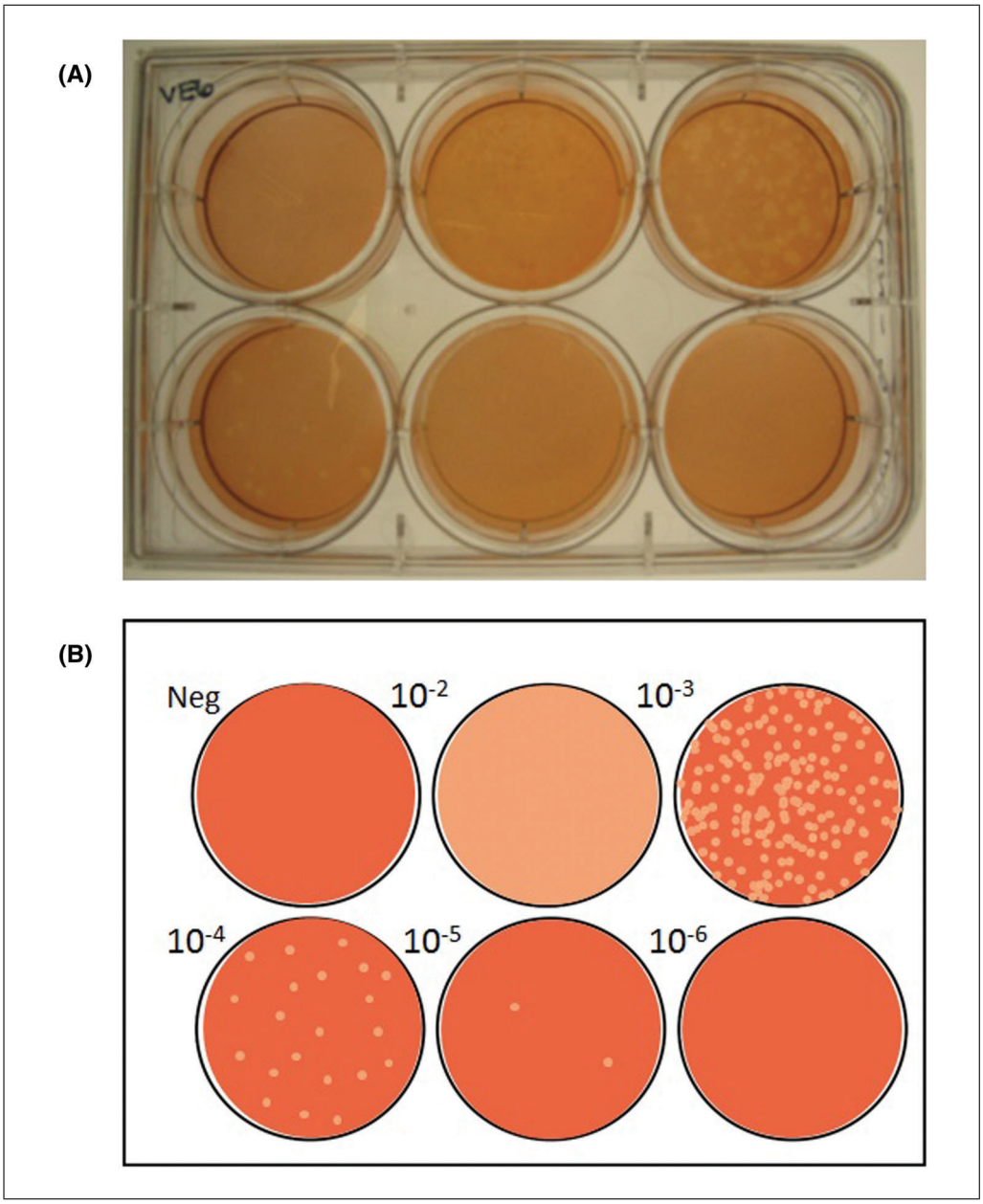

Figure 3 (**A**) Representative plaque assay plate processed by the Basic Protocol, which uses a solid double overlay method. (**B**) Schematic example of a 6-well SARS-CoV-2 plaque assay plate processed by the Basic Protocol after 3 dpi. The negative control shows an intact monolayer stained brownish red. The 10^{-6} dilution appears similar to the negative control, indicating the absence of SARS-CoV-2 plaque-forming units. The 10^{-5} and 10^{-3} dilutions show 2 and >100 peach-colored plaques, but since these values are less than 5 and greater than 100, they will not be used in the calculation of the virus titer. The 10^{-4} dilution shows 21 plaques, and thus these values will be used in the calculation of the virus titer. The titer from this plate is 2.1×10^5 PFU/ml.

10. *Titer calculation:* Identify the virus dilution factor that yields 5-100 plaques per well (Baer & Kehn-Hall, 2014). Use **Formula 7** to calculate the average number of plaques at this dilution. Use **Formula 8** to calculate the titer of SARS-CoV-2 in the specimen using the identified dilution factor and the inoculum volume of 0.1 ml. Refer to Figure 3B for an example plaque assay plate and interpretation.

    Formula 7: Average number of plaques given by the identified virus dilution = Sum of plaques from 4 replicate wells ÷ 4

Formula 8: Titer of SARS-CoV-2 (in PFU/ml) = Average number of plaques given by the identified virus dilution ÷ (dilution factor × 0.1 ml)

11. *Waste disposal:* Storage of plates prior to enumeration is not recommended because the borders of the plaques become less distinct over time. Wells of each plate can be topped up with an appropriate disinfectant (such as a 5% Micro-Chem Plus solution) and incubated at room temperature overnight prior to disposal in biohazardous waste. All biohazardous waste should be autoclaved at 121°C for 1 hr.

ALTERNATE PROTOCOL

SARS-CoV-2 PLAQUE ASSAY USING A LIQUID OVERLAY AND FIXATION-STAINING METHOD

This protocol can be used as an alternative to the Basic Protocol for the quantification of SARS-CoV-2 by plaque assay. The first steps in the protocol are identical to the Basic Protocol. However, after adsorption, a liquid overlay medium (L-OM) is applied to the cell monolayer to restrict virus growth to the originally infected foci of cells. In addition, instead of incorporating a vital stain into the secondary overlay (S-OM), the L-OM is removed from the monolayer, fixed, and stained with crystal violet, which binds to proteins and DNA within cells (Feoktistova, Geserick, & Leverkus, 2016). As a result, clear plaques can be distinguished from the purple monolayer. Plaques are enumerated and used for the calculation of virus titers in PFU/ml. For a comparison of the two protocols, see Commentary.

Additional Materials *(also see Basic Protocol)*

3% carboxymethylcellulose (CMC; (liquid matrix, see recipe)
10% neutral-buffered formalin (Sigma-Aldrich #HT501128-4L); Thermo Scientific™ Paraformaldehyde Solution, 4% in PBS (ThermoFisher Scientific #AAJ19943K2) can also be used.
Formalex GREEN Formalin Neutralizer (Jones Scientific # H-FORMG-CB)
0.5% crystal violet (CV, see recipe)

Cell culture (Day 0)

1. Perform steps 1-2 of the Basic Protocol.

Specimen dilution, infection of cells, and primary overlay (Day 1, 0 dpi)

2. Perform steps 3-4 of the Basic Protocol

3. *Preparation of L-OM:* Incubate overlay diluent at 44°C in a water bath. After rocking plates for the last time (approximately 10 min before incubation is over), prepare L-OM by adding equal amounts (1:1) of overlay diluent and 3% CMC (liquid matrix). Use **Formula 1** to calculate the total volume of L-OM needed, followed by **Formula 2** to calculate the overlay diluent and 3% CMC (liquid matrix) needed to prepare the L-OM.

   Formula 1: Total volume of L-OM needed (in ml) = Number of specimens × 4 plates per specimen × 6 wells per plate × 3 ml L-OM per well × 1.2 (to account for pipetting error)

   Formula 2: Volume of overlay diluent or 3% CMC (liquid matrix) needed for L-OM (in ml) = total volume of L-OM needed ÷ 2

4. *Liquid overlay:* Add 3 ml of L-OM to each well of the titration plates (see Basic Protocol, step 4). Incubate at 37°C in a 5% CO_2 incubator for 3 days.

   During this incubation, avoid movement of the plates, which can disrupt the liquid overlay and cause plaques to smear.

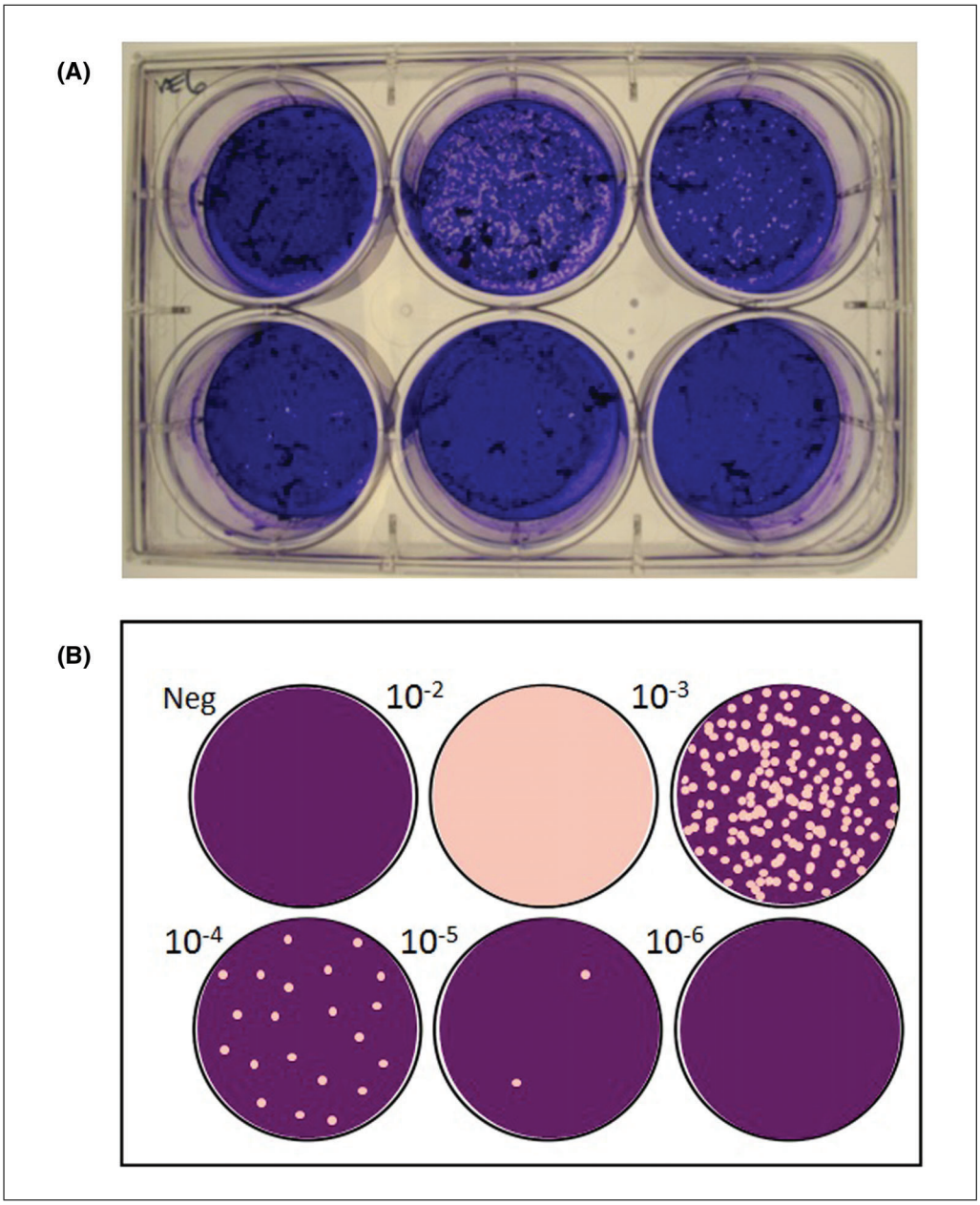

Figure 4 (**A**) Representative plaque assay plate processed by the Alternate Protocol, which uses a liquid overlay and fixation-staining method. (**B**) Schematic example of a 6-well SARS-CoV-2 plaque assay plate processed by the Alternate Protocol after 3 dpi. The negative control shows an intact monolayer stained purple. The 10^{-6} dilution appears similar to the negative control, indicating the absence of SARS-CoV-2 plaque-forming units. The 10^{-5} and 10^{-3} dilutions show 2 and >100 clear-colored plaques, but since these values are less than 5 and greater than 100, they will not be used in the calculation of the virus titer. The 10^{-4} dilution shows 21 plaques, and thus these values will be used in the calculation of the virus titer. The titer from this plate is 2.1×10^5 PFU/ml.

Fixation, staining, enumeration of plaques, and titer calculation (Day 4, 3 dpi)

5. *Fixation:* Carefully aspirate L-OM from each well and discard in a waste bottle containing an appropriate disinfectant (such as a 5% Micro-Chem Plus solution). Gently wash cells with DPBS and fill wells with 10% buffered formalin or 4% paraformaldehyde. Incubate at room temperature for 1 hr.

6. *Staining:* Aspirate fixative from wells and discard into a waste bottle containing the appropriate amount of Formalex. Add 200 µl of 0.5% CV to each well and incubate at room temperature for 5-15 minutes.

One volume of Formalex is used to inactivate 4 volumes of formaldehyde waste.

7. *Wash:* Wash cells 2-4 times with distilled water. Blot dry.

   At this point, plates can be surface-decontaminated with an appropriate disinfectant and stored at 4°C for up to 1 month.

8. *Enumerate plaques:* Plaques will appear as clear circles on a purple monolayer of cells (Figure 4A). The negative control should have a uniform monolayer, which can be used as a reference. Record the number of plaques observed per well at each virus dilution.

9. *Titer calculation:* Identify the virus dilution factor demonstrating 5-100 plaques per well. Use **Formula 7** (see Basic Protocol) to calculate the average number of plaques at this dilution. Use **Formula 8** (see Basic Protocol) to calculate the titer of SARS-CoV-2 in the specimen using the identified dilution factor and the inoculum volume of 0.1 ml. Refer to Figure 4B for an example plaque assay plate and interpretation.

10. *Waste disposal:* Dispose of plates in biohazardous waste and autoclave at 121°C for 1 hr. Liquid waste containing disinfectant should be incubated at room temperature overnight prior to disposal.

REAGENTS AND SOLUTIONS

Carboxymethylcellulose, 3% (w/v)

6 g carboxymethylcellulose, medium viscosity (solid; Sigma-Aldrich #C4888-500G)
200 ml distilled water
Sterilize by autoclaving at 121°C for 15 min
Store at room temperature up to 2 months

Cell maintenance medium

500 ml HyClone™ Dulbecco's Modified Eagles Medium, High Glucose with l-glutamine (ThermoFisher Scientific #SH30022LS)
50 ml FBS (ThermoFisher Scientific #16140071)
Store at 4°C up to 2 months

Crystal violet, 1% (w/v)

1 g crystal violet (solid; Certified Biological Stain; ThermoFisher Scientific #C581-25)
20 ml absolute ethanol
80 ml distilled water
Filter sterilize
Store at room temperature up to 4 months protected from light

Infection medium

500 ml HyClone™ Dulbecco's Modified Eagles Medium, High Glucose, with l-glutamine (ThermoFisher Scientific #SH30022LS)
10 ml FBS (ThermoFisher Scientific #16140071)
Store at 4°C up to 2 months

Neutral red, 0.33% (w/v)

0.33 g neutral red (solid; ACROS Organics™; ThermoFisher Scientific #AC415491000)
100 ml distilled water
Filter sterilize
Store at room temperature up to 4 months protected from light

Noble agar, 2% (w/v)

4 g Noble agar (solid; Ultrapure; ThermoFisher Scientific #AAJ1090736)
200 ml distilled water
Sterilize by autoclaving at 121°C for 15 min
Store at room temperature up to 2 months

Overlay diluent

500 ml Gibco™ MEM (2×), no phenol red (ThermoFisher Scientific #11935046)
40 ml FBS (ThermoFisher Scientific #16140071)
10 ml 200 mM l-glutamine (ThermoFisher Scientific #25030149)
10 ml 100× nonessential amino acids (NEAA; ThermoFisher Scientific #11140050)
7.5 ml 100× sodium bicarbonate (ThermoFisher Scientific #25080094)
Store at 4°C up to 2 months

COMMENTARY

Background Information

The accurate quantification of infectious virus specimens is crucial for a multitude of applications in virology. Plaque assays were established in 1952 as an adaptation of phage assays, which were used to calculate bacteriophage titers in plant biology (Cooper, 1961; Dulbecco & Vogt, 1953). Although new techniques for viral titration continue to evolve, plaque assays continue to be the gold standard for the quantification of infectious virus (Baer & Kehn-Hall, 2014).

The plaque assay using the double overlay method (Basic Protocol) described in this article has been adapted for SARS-CoV-2 from previously characterized procedures (Berry et al., 2004; Harcourt et al., 2020; Ströher et al., 2004). Noble agar, the solid matrix used in the overlay in the Basic Protocol, is considered a traditional overlay matrix for plaque assays. However, it comes with disadvantages. Heating is required to liquefy the matrix immediately prior to application, which warrants the need to work quickly when applying this type of overlay since it can re-solidify as it cools. The secondary overlay in this protocol uses neutral red to stain the monolayer and enhance the visualization of plaques. As a vital stain, neutral red has the advantage of being applied during incubation, enabling live monitoring of plaque formation. However, the contrast between plaques and viable cells stained with neutral red is not as distinct as that produced by crystal violet and fixation in the Alternate Protocol, and requires a light box to further enhance visualization.

The plaque assay using the fixation and staining method (Alternate Protocol) described in this article has been adapted for SARS-CoV-2 from previously characterized procedures (Schneider et al., 2012; Vicenzi et al., 2004; Wang, Sakhatskyy, Chou, & Lu, 2005). In contrast to the Basic Protocol, this method uses a liquid overlay matrix, which confers benefits over solid/semi-solid overlay matrices, including the ease of removal prior to fixation. In addition, liquid overlays can be applied to cell monolayers at room temperature, eliminating issues of re-solidification during the application process. However, the disadvantage of the liquid overlay matrix is that disruption of the liquid overlays can result in smeared plaques, and thus great care must be taken to prevent movement of plates during the incubation period. The fixation and subsequent staining with crystal violet used to enhance plaque visualization in the protocol has several advantages, including increased contrast between plaques from the purple monolayers, as well as the inactivation of the virus. In addition, fixation enables plates to be stored and plaques to be enumerated at a later time. However, some disadvantages of this method includes the generation of formaldehyde waste and the additional need to properly inactivate that chemical.

As summarized in Table 1, each protocol has advantages and disadvantages that can be assessed to select the method best suited to each laboratory's resources and preferences. A comparison conducted in our laboratory demonstrated that there were no significant differences in viral titers calculated using either method.

Critical Parameters

Since SARS-CoV-2 is a BSL-3 pathogen, procedures using SARS-CoV-2 specimens require BSL-3 facilities, equipment, and operational practices. Additional personal protective equipment (PPE), including

Table 1 Advantages and Disadvantages of SARS-CoV-2 Plaque Assays using a Solid Double Overlay Method (Basic Protocol) and Liquid Overlay and Fixation-Staining Method (Alternate Protocol)

Characteristic	Basic Protocol	Alternate Protocol
Completion time	Completed in 4 days (cell culture excluded)	Completed in 4 days (cell culture excluded)
Number of steps	**Advantage**: Neutral red applied in secondary overlay bypasses need for additional washes, fixation, and staining steps	Disadvantage: Additional washing, fixation, and staining steps needed
Work in BSL-3 lab	Disadvantage: Secondary overlay to stain cells requires manipulation of plates the day before enumeration (assay needs 3 days working in BSL-3)	**Advantage**: Fixation, staining, and enumeration on same day instead of different day (assay only needs 2 days working in BSL-3 lab)
Overlay application	Disadvantage: Requires speed to apply solid overlay to monolayers to avoid re-solidifying	**Advantage**: Liquid overlay easily applied
Incubation	**Advantage**: Plaques in solid overlay not prone to distortion if plates are moved	Disadvantage: Movement of plates during incubation can disrupt liquid overlay, causing plaques to appear as streaks
Additional chemicals needed	**Advantage**: Does not require fixation step	Disadvantage: Fixation step requires formaldehyde fixatives and chemicals used for inactivation of formaldehyde waste
Additional equipment needed	Disadvantage: Requires a microwave oven to dissolve agar and light box to enhance visualization of plaques	**Advantage**: Overlay in liquid form
Visualization	Disadvantage: Peach-colored plaques less distinct from brownish-red monolayer	**Advantage**: Clear plaques very distinct from purple monolayer
Storage of plates	Disadvantage: Plaques must be enumerated within a few days of secondary overlay	**Advantage**: Plates can be stored at 4°C to read plaques up to a month
Cost	**Advantage**: Less reagents and consumables used per assay	Disadvantage: Additional reagents and consumables used per assay
Safety precautions during enumeration	Disadvantage: Virus is still viable and infectious while reading plates	**Advantage**: Virus is inactivated during fixation step

respiratory protection, is required for working with SARS-CoV-2 (WHO, 2020b). It is crucial to consult with on-site biosafety officers, occupational hygienists, and other related personnel to conduct risk assessments and provide guidance regarding protective equipment, effective disinfectants, and practices to mitigate risks when working with the pathogen.

The monolayer is susceptible to damage from forceful pipetting, which can then be mistaken for plaques. Instead of adding medium directly on top of the monolayer, slowly add it by aiming for the walls of each well.

Troubleshooting

See Table 2 for commonly encountered problems, causes, and solutions. A plaque assay plate exhibiting commonly encountered problems is depicted in Figure 5.

Understanding Results

Refer to Figure 3 for an example interpretation of a schematic 6-well SARS-CoV-2 plaque assay plate. In our laboratory, we have found that the titers of most of our SARS-CoV-2 stocks passaged in Vero E6 and titrated by plaque assays using Vero E6 range from 10^5 to 10^6 PFU/ml.

Table 2 Troubleshooting Guide for Basic Protocol and/or Alternate Protocol

Problem	Possible cause	Solution
Plaque numbers >100 at all dilutions	Virus titers of the specimen may be greater than 10^7 PFU/ml	Conduct another plaque assay with further serial dilutions of the specimen (i.e., 10^{-7}, 10^{-8}, 10^{-9}, etc.)
	Improper technique for serial dilutions	Make sure to change pipet tips, vortex well, and briefly centrifuge between dilutions
Plaque numbers <5 at all dilutions	Virus titers of the specimen may be less than 100 PFU/ml	Conduct another plaque assay and infect cells with the undiluted sample, as well as the sample diluted 10^{-1}
	Heat from the S-OM (Basic Protocol) could possibly affect infectivity of SARS-CoV-2	Make sure S-OM cools slightly from 44°C before applying it to cell monolayer
Plaques clustered around edges of wells	Swirling the inoculum during the adsorption stage	Use a rocking motion from front to back and side to side to distribute the inoculum evenly on the cell monolayer
Large area of missing cells on monolayer	Overlay medium or other reagents added quickly and directly on top of monolayer	Add medium slowly and aim for edge of well
	Cells not confluent prior to start of assay	Use a light microscope to check that cells are at least 95% confluence before starting assay
"Shooting star" or smear-like appearance of plaques (Alternate Protocol)	Liquid overlay disrupted during incubation	Minimize movement of plates during incubation by leaving plates untouched throughout the incubation and reducing the number of times the incubator door is opened and closed.
"Crescent moon" along edge of well	Drying of cell monolayer	(a) Leave ~50-100 µl of liquid in each well when aspirating medium and DPBS; (b) rock plates every 10-15 min during adsorption incubation; (c) work in small batches of plates to prevent the monolayer from drying out between each aspiration and medium addition
Large plaque-like spot	Scratching of the monolayer by pipette tip during medium addition or removal	Avoid making touching the monolayer with pipette tip by hovering above the well and aiming along the edges when adding medium. Minimize the force used when using a serological pipette tip to remove medium.
	Medium added directly to monolayer at high speed	Aim medium along the edges at low speed

Time Considerations

The Basic Protocol and Alternate Protocol can both be completed in 5-7 days, including the time needed to seed plates for the plaque assay. However, if starting Vero E6 cells from cryostocks, it would be necessary to passage the cell line at least three times prior to use in either assay to remove excess cryopreservative and allow cells to return to normal growth, extending the duration by a few days; this is not taken into account in the protocols. If plates are seeded as described in the protocols on a Monday, the plaque assay can begin on Tuesday and end with plaque enumeration on Friday. Using this schedule, the secondary overlay would be applied on Thursday for the Basic Protocol.

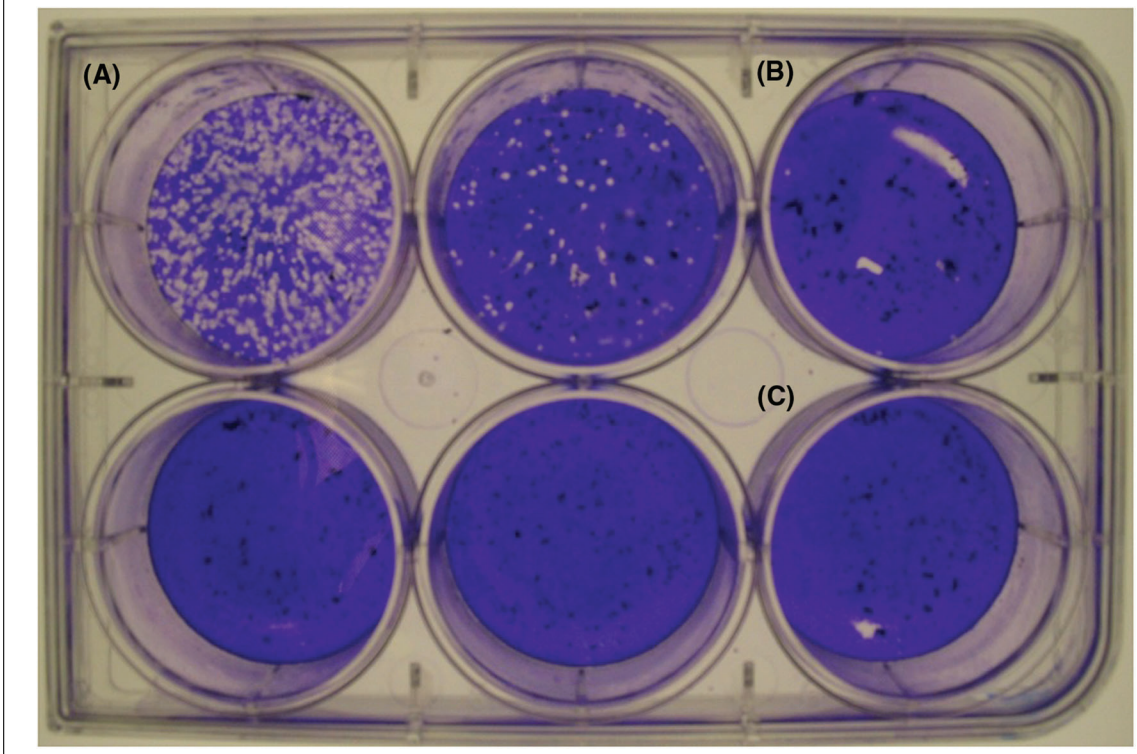

Figure 5 A representative plaque assay plate exhibiting commonly encountered problems. (**A**) "Shooting star" appearance of plaques in the first two wells is encountered when the liquid overlay is disrupted during the incubation. (**B**) "Crescent moon" along edge of wells can occur if the cell monolayer is allowed to dry upon aspirating cell culture medium/DPBS or if inoculum is not evenly distributed across the monolayer by rocking during adsorption. (**C**) A large plaque-like spot that differs from the other plaques in size, morphology, and border distinction can occur when the monolayer is scratched by a serological pipette tip or medium is added directly to monolayer at high speed.

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