bio-protocol

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Supplementary information for A Plaque Assay to Determine the Titer of a Recombinant Live-Attenuated VSV Vaccine for SARS-CoV-2: Method Development and Validation

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Supplementary Protocol 1: Preparation of Vero E6 cell plates for the plaque assay

This procedure also serves as a record. Mark √ next to each completed item and enter the required details on the blank lines.

A. General

This procedure describes the preparation of plates for determination of virus titer by the PFU assay.

B. Responsibilities

Personnel must be proficient in cell-culture work and qualified to prepare plates for determination of virus titer by the PFU assay.

C. Safety

Work shall be performed in accordance with the institute safety procedures for work with cell cultures.

D. Equipment

1. Incubator, 37 °C, 5% CO_2, with humidified atmosphere
2. Class II biosafety cabinet
3. T-150 cell-culture flasks

4. Disposable plastic pipettes, 2, 5, 10, and 25 mL
5. Cell-counting instrument
6. Microscope
7. Sterile 15 or 50 mL tubes
8. 6-well plates

E. Materials and solutions

Prior to use, verify that all materials and solutions are within their approved expiry dates.
1. Vero E6 ATCC WCB cells (650,000–750,000 cells per well), passage number up to 19
2. 0.5% Trypan Blue solution
3. Trypsin
4. 1× PBS
5. Cell-seeding medium containing DMEM + 10% FBS + 2 mM L-glutamine + 1% non-essential amino acids + 0.5% Pen/Strep

F. Procedure

Details of the cells selected for the assay:
1. Passage number: _____. Split date for this passage: _____. Split ratio: 1:____. The cells have typical morphology and are suitable for seeding.
2. The most recent split was performed no more than two days before the seeding date.

Upon reaching passage 15, thaw a new vial of Vero E6 ATCC WCB cells.

Calculation of the number of cells and the volume of medium required for an assay using a specified number of plates

Table 1. Cell number and medium volume data

_____	A	Number of plates required for the assay
_____	B = A + 2	Number of plates to prepare, including excess
_____	C = B × 6	Number of wells
	650,000–750,000	Number of cells per well
_____	D = C × 700,000	Number of cells required for seeding
_____	E = C × 3	Volume of medium (mL) in which the cells will be resuspended before plate seeding
_____	N = (B / 4) + 2	Number of flasks from which cells must be harvested

Approver name: _____ Signature: _____

Cell harvesting

Process no more than two flasks simultaneously per operator and no more than six flasks per operator during the entire procedure.

1. Wash the cells with 5 mL of PBS.
2. Add 5 mL of trypsin and incubate for 5 min at 37 °C.
3. Tap the flask to detach the cells.

4. Add 20 mL of fresh medium.
5. Pipette the liquid up and down 5–10 times using a 10 mL pipette to eliminate cell clumps.
6. Pool the contents of the flasks in an Erlenmeyer flask.
7. Mix and withdraw two separate 0.2 mL samples. Count each sample using a counting chamber.

Table 2. Cell concentration

Viability (%)	**Concentration (cells/mL)**	
		Sample 1
		Sample 2
	X = _____	Mean*

*If the difference between the two samples is greater than 20%, collect and count a third sample.

Table 3. Calculation of the cell suspension volume for seeding and the volume of medium required for adjustment

_____	D / X	Required volume of cell suspension for seeding (mL)
_____	E – D / X	Volume of medium (mL) to add to the cell suspension before plate seeding

1. A volume of _____ mL cell suspension was added to _____ mL medium and mixed.
2. Slowly dispense 3 mL of cell suspension into each well. Before each aspiration with the pipette, gently mix the cell suspension in the tube. After filling each plate, move it gently from side to side and backward and forward.
3. Incubate the plates for 22–30 h in a 5% CO_2 incubator.
4. Approximately 1 h after placing the plates in the incubator, gently move each plate from side to side and backward and forward.

G. Transfer of the cell plates to the PFU assay

Before transferring the plates, verify the following:
1. The cells have morphology typical of this culture.
2. Confluency ≥80% at the time of plate transfer from the cell-culture facility (reaching 90%–100% confluency at the time of viral inoculation).
3. No cell clusters are present in the center of the plate.
4. Few cells are present in the supernatant.

The plates were inspected and transferred by _____, to _____, date and time: _____.

Supplementary Protocol 2: Plaque assay procedure

A. General

This work instruction is intended for the determination of the titer of the rVSV-SARS-CoV-2-S virus by a plaque-forming unit (PFU) assay in Vero E6 cell culture, using drug product (DP) samples for which the target titer is known; therefore, an appropriate dilution series is selected according to this titer. Each DP sample is tested at two time points. A separate batch record (BR) must be completed for each test time point. The final result will be calculated as the average of the results obtained at the two time points.

B. Responsibilities

Personnel must be proficient in cell-culture work and in titration of plaque-forming viruses in tissue culture and must be qualified to perform the determination. Results shall be approved by a QC manager or by an authorized designee.

C. Safety

The required biosafety level for this work is BL2. The work shall be performed in accordance with the institute safety procedures for work with the rVSV-SARS-CoV-2-S virus. All work with the virus shall be performed in a Class II biological safety cabinet. A laboratory coat, gloves, and safety glasses shall be worn.

DP batch/sample description: _____
Test time point documented in this procedure: First time point / Second time point (circle the applicable option).

D. Equipment

1. Class II biosafety cabinet
2. Controlled incubator, 37 ± 2 °C, 5% CO_2, with humidified atmosphere
3. Tube mixer (vortex)
4. Sterile 4–5 mL tubes (for virus dilutions)
5. Multipette plus dispenser
6. Pipettors with volumes up to 1,000 µL; verify calibration validity before use
7. Sterile 50 mL tubes
8. Eppendorf Combitips Plus, 50 mL
9. Eppendorf Combitips Plus, 10 mL
10. Sterile 250 mL plastic cups for preparation of the overlay medium
11. Sterile 13 mL tubes (for virus dilutions exceeding 4 mL)
12. Sterile pipettes, 5, 10, and 50 mL
13. Sterile tips with volumes up to 1,000 µL
14. Ice and timer
15. Rocker (inside the incubator)
16. Liquid-aspiration system with a disinfectant trap inside the cabinet
17. Pipette controller, Pipet-Aid or equivalent
18. Magnifying glass and marking pen

E. Materials and solutions

Prior to use, verify that all materials and solutions are within their approved expiry dates.

Cell monolayer: 6-well tissue-culture plates containing a Vero E6 cell monolayer, the preparation of which is documented in the cell-culture laboratory.
Cell passage number: _____. Plates prepared on: _____, according to the relevant procedure.

Solutions
1. Dilution medium, preparation documented in the relevant procedure
2. Tested and approved tragacanth overlay medium.
3. 0.1% crystal violet staining solution.
4. QC sample: ____, with a mean titer of _____ PFU/mL (acceptable range: ±0.5 $\log_{10}$).

F. Procedure

Note: Disinfect the inside of the cabinet before beginning work by wiping with alcohol or a bleach solution.

1. Dilute the test samples for inoculation into 6-well tissue-culture plates to concentrations of 75, 150, and 300 PFU/mL. Inoculation of 200 µL per well will therefore yield approximately 15, 30, and 60 PFU/well, respectively. Calculation of the

virus titer in the original sample shall be based only on wells containing 10–80 PFU/well, in which the plaques can be counted reliably.
2. The DS sample from which the final product was prepared shall be tested on the day of formulation, and the test shall be documented in the procedure.
3. The DP titer shall be determined at two time points. The two time points represent two independent assay runs performed on different days (typically separated by at least 24 h and both conducted within 1 week of freezing the DP batch). At each time point, the mean titer shall be determined from three different frozen vials that are thawed within one week of freezing. The assays shall be performed using three two-fold dilutions, as described above, with six replicate wells per dilution for each vial. For each vial, the mean shall be calculated from at least two dilutions (at least four wells per dilution).
4. Calculation of the virus concentration in the DP shall be based on the mean of the three frozen vials at each time point and on the mean of the two different time points.
5. Total number of plates required for determination of the DP titer after thawing: twelve 6-well tissue-culture plates.
6. Dilute the QC sample according to its known expected titer in three independent replicates, and inoculate one dilution onto three plates, six wells per plate. Up to two wells per plate may be excluded.
7. Leave two wells as negative controls (no virus infection) and add 0.2 mL dilution medium to each.
8. Dispose of excess samples upon completion of the work.

G. Preparation of dilutions and virus inoculation

1. Remove frozen test samples (including the QC sample) from the freezer and thaw at room temperature until completely liquid (do not refreeze). Mix the thawed stock gently by inversion or gentle swirling (5–10 times) to avoid foaming or excessive shear.
2. Prepare dilution tubes containing dilution medium according to the volume required for inoculation, as specified in the dilution table.
3. Label the plates with sequential numbers on both the side and the lid and mark the test samples and prepared dilutions on the plate lids.
4. Record the arrangement of the samples and prepared dilutions in Table 2.
5. Aspirate the growth medium from the cell monolayers, leaving approximately 0.2 mL in each well.
6. Inoculate each well with 0.2 mL of each test sample and of the QC sample.
7. Inoculate the samples into the plates as specified under “General notes and instructions”: QC-sample dilutions according to Table 1 and test-sample dilutions according to Table 2.
8. Incubate the plates for 1 h in a controlled incubator at 37 ± 2 °C and 5% CO_2, with a humidified chamber, incubator no. _____, with rocking from _____ to _____.
9. Add 3 mL of overlay medium to each well, and incubate the plates in a controlled incubator at 37 ± 2 °C and 5% CO_2, with a humidified chamber, incubator no. _____, from _____ to _____, for 72 h.

Table 1. QC sample dilutions

Dilution	Virus volume	Dilution-medium volume
1:10	0.05 mL stock	0.45 mL
1:100 (1:10 + 1:10)	0.1 mL	0.9 mL
$1:10^3$ (1:100 + 1:10)	0.1 mL	0.9 mL
$1:10^4$ ($1:10^3$ + 1:10)	0.1 mL	0.9 mL
5×10^4 ($1:10^4$ + 1:5); 1:50,000	0.3 mL	1.2 mL

The QC sample was finally diluted _____ (1:50,000), to an expected concentration of 260 PFU/mL.
Each test sample was diluted according to the dilution plan described in Table 2 (based on the expected titer). The samples were mixed by vortexing between dilution steps.

Table 2. Preparation of the DP sample dilutions for concentrations in the range of 1×10^7 to 5×10^8 PFU/mL

Final dilution	Dilution preparation	Virus volume	Dilution-medium volume
10^1	1:10	0.1 mL (stock)	0.9 mL
10^2	1:100 (1:10 + 1:10)	0.1 mL	0.9 mL
10^3	$1:10^3$ (1:100 + 1:10)	0.1 mL	0.9 mL
10^4	$1:10^4$ ($1:10^3$ + 1:10)	0.2 mL	1.8 mL

10^4 (1:5)	1:5 × 10^4 (1:10^4 + 1:5)	0.3 mL	1.2 mL
10^5	1:10^5 (1:10^4 + 1:10)	0.2 mL	1.8 mL
10^5 (1:1)	1:2 × 10^5 (1:10^5 + 1:2)	1.0 mL	1.0 mL
10^5 (1:5)	1:5 × 10^5 (1:10^5 + 1:2.5)	0.3 mL	1.2 mL
10^6	1:10^6 (1:5 × 10^5 + 1:2)	0.2 mL	1.8 mL
10^6 (1:1)	1:2 × 10^6 (1:10^6 + 1:2)	0.7 mL	0.7 mL
	*		

*Additional optional dilution (as instructed by the QC manager).

H. Plate development by staining and plaque counting

1. Transfer the plates to the cabinet and aspirate the overlay medium as completely as possible.
2. Stain the wells by adding 1 mL of 0.1% crystal violet staining solution to each well.
3. Leave the stain for 4–6 min at room temperature in the cabinet.
4. Aspirate the staining solution from the wells and gently rinse the cell monolayers with tap water.
5. Count the plaques and document in Table 3.

Table 3. Plaque counts in the plates (raw results)

Plaque count						Dilution	Test sample	Plate No.	Vial No.
									1
									2
									3

Table 4. QC sample plate counts (at least four wells per plate)

Plaque count						Dilution	Plate No.
						1:50,000	1
						1:50,000	2
						1:50,000	3

I. Entry and storage of results

1. Calculate the titer, in PFU/mL, from the data in Table 3. Calculations are performed using Microsoft Excel in a dedicated protected file named: _____.
Save the file in: P – GENERAL – Department Analyses – PFU.
2. A signed calculation file was appended to this work instruction. Number of attached pages: _____

J. Processing of results (according to the Excel file)

Record the calculated titer in Table 5 according to the results obtained in the relevant Excel file.

Table 5. Calculated titer

Mean	Calculated titer (PFU/mL)	Test sample
		QC sample
		DP, vial 1
		DP, vial 2
		DP, vial 3
		DP, final titer

K. Criteria for titer calculation and assay validity

Calculation of sample titers

1. Sample titers shall be calculated using Microsoft Excel and the dedicated "DP Template." The calculation area in this file is locked and cannot be modified. Record the file name in the designated location (Section I.1). The calculation shall include the mean, standard deviation (STDEV), coefficient of variation (%CV), and confidence interval (C.I.). The file shall be saved as described in Section I.1.

2. Sample-titer calculations in the computerized file shall be performed as follows:
a. Calculate the mean and standard deviation of the plaque count for each dilution, provided that the count is between 10 and 80 plaques per well.
b. Wells exceeding ±30% CV shall not be included in the calculation of the mean.
c. The %CV shall be calculated from all wells meeting the criterion in the preceding section.
d. If the %CV after removal of outliers exceeds 30%, the values for that dilution shall not be used.
e. The results from the relevant wells shall be used for the calculation and evaluated against the applicable acceptance criteria (C.I.).
f. The final mean and standard deviation for the dilution shall be calculated from the relevant wells and entered in the summary table.

3. The QC sample titer must be within its established range (between the upper and lower limits defined according to the control chart). In addition, the QC sample titer must meet the acceptance criteria in the titer-calculation file, including the C.I. calculation.
4. Counts below 10 PFU shall be marked TL (too low), and counts above 80 PFU shall be marked TH (too high); these counts shall not be included in the titer calculations.

L. Assay acceptance/rejection criteria

An invalid assay is defined as an assay in which:
1. The confidence interval (C.I.) of the QC sample determination in the assay is greater than ±0.5 $\log_{10}$ of the mean value obtained in this assay (Log) _____.
The permitted C.I. range for the QC sample in this assay (mean ± 0.5 $\log_{10}$): (Log) _____ – (Log) _____.
The C.I. range of the QC sample obtained in this assay: (Log) _____ – (Log) _____.
This value is / is not within the range (circle the applicable option).

2. The value determined for the QC sample in the assay is 0.5 $\log_{10}$ higher than its mean assigned value. The value obtained in this assay is _____ PFU/mL.
The permitted titer range for the QC sample is: _____ – _____ PFU/mL.
The QC sample titer obtained in this assay is / is not within the range (circle the applicable option).

3. The confidence interval (C.I.) for each test sample is within the range calculated in the attached worksheet. The value determined in the assay is greater than 0.5 $\log_{10}$.
4. The determined value _____ does not meet the expected range. The confidence interval (C.I.) between the two time points is within the range calculated in the attached worksheet. The value determined in the assay is greater than 0.5 $\log_{10}$.

Negative control

Wells with an intact, normal monolayer and uniform staining: Yes / No.

The assay is acceptable / The assay is not acceptable (circle the applicable option)
DP sample from batch: _____ meets / does not meet these conditions.