

## **Evaluation of Neutralizing Antibodies against Highly Pathogenic Coronaviruses: A Detailed Protocol for a Rapid Evaluation of Neutralizing Antibodies Using Vesicular Stomatitis Virus (VSV) Pseudovirus-based Assay**

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## **ABSTRACT**

Emerging highly pathogenic human coronaviruses (CoVs) represent a serious ongoing threat to the public health worldwide. The spike (S) proteins of CoVs are surface glycoproteins that facilitate viral entry into host cells via attachment to their respective cellular receptors. The S protein is believed to be a major immunogenic component of CoVs and a target for neutralizing antibodies (nAbs) and most candidate vaccines. Development of a safe and convenient assay is thus urgently needed to determine the prevalence of CoVs nAbs in the population, to study immune response in infected individuals, and to aid in vaccines and viral entry inhibitors evaluation. While live virus-based neutralization assays are used as gold standard serological methods to detect and measure nAbs, handling of highly pathogenic live CoVs requires strict bio-containment conditions in biosafety level-3 laboratories. On the other hand, use of replication-incompetent pseudoviruses bearing CoVs S proteins could represent a safe and useful method to detect nAbs in serum samples under biosafety level-2 conditions. Here, we describe a detailed protocol of a safe and convenient assay to generate vesicular stomatitis virus (VSV)-based pseudoviruses to evaluate and measure nAbs against highly pathogenic CoVs. The protocol covers methods to produce VSV pseudovirus bearing the S protein of the Middle East respiratory syndrome-CoV (MERS-CoV) and the severe acute respiratory syndrome-CoV-2 (SARS-CoV-2), pseudovirus titration, and pseudovirus neutralizing assay. Such assay could be adapted by different laboratories and researchers working on highly pathogenic CoVs without the need to handle live viruses in biosafety level-3 environment.

## **KEYWORDS**

Coronaviruses; MERS-CoV; SARS-CoV-2; Serological assay; VSV pseudovirus

## INTRODUCTION

Coronaviruses (CoVs) are the largest group of enveloped positive-sense RNA viruses that primarily infect the respiratory and gastrointestinal tracts of birds and mammals (Fehr and Perlman, 2015). Many CoVs could be zoonotic viruses capable of crossing the species barrier and infecting humans (Kandeel et al., 2020). Human CoVs mainly cause mild respiratory tract infections and no highly pathogenic CoVs were recognized until the beginning of the 21<sup>st</sup> century (Cui et al., 2019). Since 2002, a number of highly pathogenic human CoVs have emerged including the severe acute respiratory syndrome-CoV (SARS-CoV) in 2002/2003 and the Middle East respiratory syndrome-CoV (MERS-CoV) in 2012 (Ksiazek et al., 2003, Zaki et al., 2012). In December 2019, a novel human highly pathogenic CoV known as severe acute respiratory syndrome-CoV-2 (SARS-CoV-2) has emerged in Wuhan, China causing the coronavirus disease-19 (COVID-19) pandemic (Wu et al., 2020, Zhu et al., 2020). While SARS-CoV has disappeared, both MERS-CoV and SARS-CoV-2 continue to be a major global threat especially that no clinically proven treatments or vaccines are available for human use to date (Al-Amri et al., 2017, Liu et al., 2018, Padron-Regalado, 2020, Ma et al., 2020, Tse et al., 2020).

The spike (S) proteins of CoVs are surface glycoproteins that facilitate viral entry into host cells. The S1 subunit at the N terminal end of the S protein contains the receptor-binding domain (RBD) responsible for the attachment to cellular receptors, while the S2 subunit at the C-terminus contains the necessary machinery that mediates the fusion with the host membranes. The S protein of SARS-CoV and SARS-CoV-2 attaches to the angiotensin-converting enzyme 2 (ACE2) receptor, and the S protein of MERS-CoV recognizes the dipeptidyl peptidase 4 (DPP-4) (Liu et al., 2018, Hoffmann et al., 2020). The S protein, particularly the RBD, is considered as a major immunogenic component of CoVs and a target for most neutralizing antibodies (nAbs).

Live virus-based neutralization methods are the gold standard serological assays to detect and measure nAbs levels, however, they require working under strict biocontainment conditions in biosafety level-3 (BSL-3) laboratories when working with highly pathogenic CoVs (Algaissi and Hashem, 2020). While conventional enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) have

been used for CoVs antibodies screening, cross-reactivity with other common CoVs may lead to false-positive results (Lester et al., 2019, Degnah et al., 2020). Additionally, positivity in these assays does not necessarily reflect the presence of nAbs in samples, requiring other confirmatory functional assays. Thus, replication-incompetent pseudotyped viruses bearing S proteins from highly pathogenic CoVs could represent an alternative safe and convenient method for CoVs nAbs detection and quantification in serum samples under biosafety level-2 (BSL-2) conditions (Almasaud et al., 2020). Several studies have shown encouraging results by utilizing vesicular stomatitis virus (VSV) as a platform to generate pseudoviruses that can be used in seroepidemiological studies, vaccine development, monoclonal antibodies and entry inhibitors screening, and basic research investigations of CoVs (Fukushi et al., 2005, Lester et al., 2019, Nie et al., 2020).

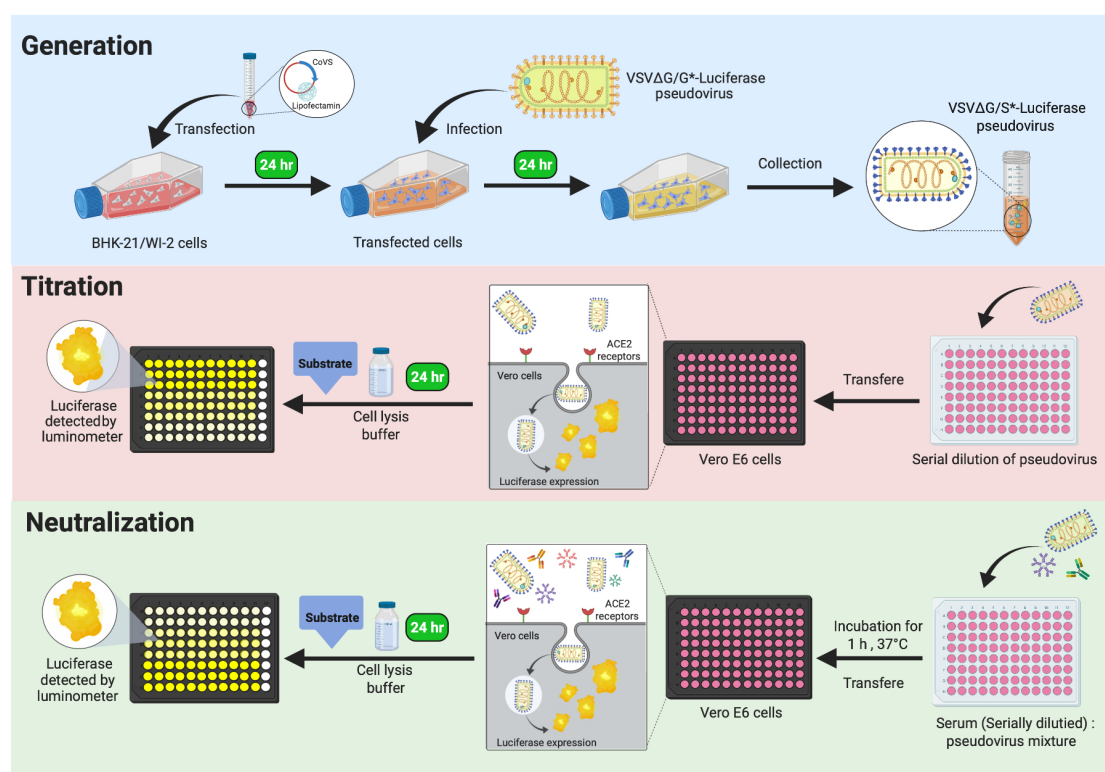
VSV is a zoonotic enveloped negative-stranded RNA virus that infects a wide range of animals and less frequently humans causing mild flu-like illness symptoms (Rodriguez, 2002, Tani et al., 2011). Its small 11 kb genome, simple structure, and ability to grow in different types of mammalian cells with high-titer made VSV a promising virus vector and a valuable tool in molecular biology and virology fields (Ruedas and Connor, 2017). Interestingly, recombinant VSV (rVSV) with G gene being replaced by reporter luciferase gene (rVSV- $\Delta$ G-luciferase) can normally bud from cells transfected with mammalian expression plasmid encoding VSV G protein or heterologous envelope protein from other viruses (Whitt, 2010, Tani et al., 2011). Thus, rVSV- $\Delta$ G-luciferase system could be used to produce single round replication-incompetent VSV pseudoviruses bearing any viral envelope glycoprotein specially from those requiring work under BSL-3 containment in BSL-2 laboratories (Whitt, 2010).

In these detailed protocols, we explain step by step how to generate VSV pseudoviruses bearing CoVs S proteins from MERS-CoV and SARS-CoV-2 using transient expression in BHK-21/WI-2 cells (Basic Protocol 1). This is followed by pseudovirus titration method (Basic Protocol 2) and pseudovirus-based neutralization assay (Basic Protocol 3) relying on reading quantitative luciferase luminescence signals. We utilized this system to conduct seroprevalence studies and measure nAbs in MERS-CoV and SARS-CoV-2 infected patients. Notes and comments have been

added to overcome any difficulties. We believe that the described platform (Figure 1) can be adapted and used for research studies as well as diagnostic purposes.

To maintain the sterility and ensure success in all cell culture experiments in all basic protocols listed here, some precautions have to be taken, which include:

- All experiments related to cell culture should be performed in BSL-2 cabinet in a tissue culture room under suitable antiseptic techniques.
- Sterile materials and solutions must be used throughout the protocol.
- All solutions should be warmed up in 37°C water bath or room temperature as specified in the protocol.
- All cells growth and incubation should be carried out in a humidified 37°C incubator with 5% CO<sub>2</sub>.



**Figure 1. Graphical overview of Basic protocols 1, 2 and 3.** Basic Protocol 1 (Generation of VSV pseudoviruses bearing CoV S protein), Basic Protocol 2 (Titration assay of the generated VSV pseudoviruses), and Basic Protocol 3 (Neutralization assay to determine CoVs specific nAbs titers in serum samples). Created with BioRender.com.

## Basic protocol 1

### Production of VSV pseudoviruses bearing CoV S protein

This protocol can be used to generate VSV pseudoviruses bearing S protein from either MERS-CoV or SARS-CoV-2 in BHK-21/WI-2 cell line. BHK-21/WI-2 cells are initially transfected with mammalian expression vector expressing the S protein of either viruses, and subsequently infected with rVSV- $\Delta$ G/G\*-luciferase 24 h later to produce pseudoviruses. Full length S genes based on consensus sequences were codon-optimized and cloned into pcDNA3.1 vector. Genes were cloned into the vector following standard cloning techniques. Plasmids are transformed into DH5 $\alpha$  cells or similar cells using ampicillin as selection antibiotic. High-quality purified plasmids are obtained using commercially available endotoxin-free maxiprep kit. Lipofectamine 2000 transfection reagent is used to deliver the constructed plasmid into BHK-21/WI-2 cells to express the S protein on the cells surface. After 24 h of the transfection, transfected cells are infected with rVSV- $\Delta$ G/G\*-luciferase to produce pseudotyped VSV (rVSV- $\Delta$ G/S\*-luciferase), which contains the surface glycoproteins from either MERS-CoV or SARS-CoV-2. Different mammalian expression vectors such as pCAGGS, plasmid purification kits or methods, or transfection reagents could be used in this protocol. Additionally, this system could be used to generate any pseudotyped viruses expressing the protein of interest simply by using other plasmids.

### Materials

- Baby Hamster kidney BHK-21/WI-2 cell line (Kerafast catalogue # EH1011)
- DMEM-5 cells growth medium (see recipe)
- Opti-MEM<sup>®</sup> I (1X) reduced-serum medium (Gibco catalogue # 31985062 or equivalent)
- 1x PBS (Biosera catalogue # LM-S2041/1000 or equivalent)
- 1x trypsin (Gibco catalogue # 12484-028 or equivalent)
- pcDNA-S: Mammalian expression vector expressing MERS-CoV or SARS-CoV-2 S protein
- pCAGGS-G-Kan plasmid (Kerafast catalogue # EH1017)

Lipofectamine™ 2000 transfection reagent (Invitrogen catalogue # 11668019, other transfection reagent or methods could be used as alternative if available)

rVSV-ΔG/G\*-luciferase (Kerafast catalogue # EH1020-PM)

Rabbit anti VSV-G polyclonal antibodies (commercial or in-house made, other species could be used to generate antibodies)

BSL-2 cabinet

37°C incubator with CO<sub>2</sub> (Heal Force HF90 or equivalent)

Water bath (Lab Tech LWB-111D or equivalent)

Benchtop low-speed centrifuge (Sigma 2-16P or equivalent)

Tissue culture T175 flasks with vented caps (SPL life sciences catalogue # 71175 or equivalent)

Sterile microcentrifuge tubes

Polypropylene sterile conical tubes:

15-ml (Falcon catalogue # 352099 or equivalent)

50-ml (Falcon catalogue # 352098 or equivalent)

Sterile serological pipettes

5-ml (SPL life sciences # 91005 or equivalent)

10-ml (SPL life sciences # 91010 or equivalent)

25-ml (SPL life sciences # 91025 or equivalent)

Micropipettes

Micropipette tips

10 µl filter tips, low retention (BIOLOGIX catalogue # 23-0011 or equivalent)

200 µl filter tips, low retention (BIOLOGIX catalogue # 23-0201 or equivalent)

1000 µl filter tips, low retention (BIOLOGIX catalogue # 23-1001 or equivalent)

Inverted microscope (Olympus CK30 or equivalent)

Timer

Ultra-low freezer (-80°C)

70% Ethanol; to sanitize all materials before use it inside the BSL-2 cabinet

### ***Day 1: BHK-21/WI-2 cells transfection with expression vector encoding CoV S protein***

1. In BSL-2 cabinet, passage T75 tissue culture flask of 90% confluent BHK-

21/WI-2 cells at 1:4 ratio into a T175 tissue culture flask using DMEM-5 medium one day before the transfection, and incubate the cells for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator. See Current Protocols article: Phelan and May (2015) for basic cell culture techniques in mammalian cell tissue culture.

*BHK-21/WI-2 cells confluent flask is passaged at 1:4 ratio to be 70% confluent next day.*

2. After 24 h, replace the growth medium of the plated BHK-21/WI-2 cells in the T175 tissue culture flask with 22 ml pre-warmed DMEM-5.

*Check the cells under the inverted microscope to ensure they are healthy and at the required confluency. After changing the media, return the flask to the incubator until preparing the transfection mixture.*

3. Add 1.75 ml of Opti-MEM reduced serum medium into two 15 ml sterile polypropylene conical tubes: tube A receives 46 µg of the pcDNA-S (expressing S protein from either MERS-CoV or SARS-CoV-2), while tube B receives 92 µl of lipofectamine 2000 transfection reagent.

*pcDNA expresses MERS-CoV S is used to generate VSV pseudovirus bearing MERS-CoV S (rVSV-ΔG/MERS-S\*-luciferase) and pcDNA expresses SARS-COV-2 S is used to generate VSV pseudovirus bearing SARS-CoV-2 S (rVSV-ΔG/SARS-2-S\*-luciferase). As a positive control, a T175 flask can be transfected with expression plasmid encoding VSV-G (pCAGGS-G-Kan plasmid, Kerafast Cat # EH1017) to generate rVSV-ΔG/G\*luciferase.*

4. Mix the mixture in each tube by pipetting up and down 10 times and incubate them for 5 min at room temperature.
5. Transfer the plasmid mixture in tube A into tube B and mix gently by pipetting up and down 10 times.
6. Incubate the transfection mixture for 20 min at room temperature.

*3.5 ml is the total volume of the transfection mixture.*

7. Take out the BHK-21/WI-2 cells in the T175 flask from the incubator and transfect the cells by adding the transformation mixture dropwise on the cells monolayer using 5 ml sterile serological pipette while swirling the flask gently to ensure even dispersal.
8. Incubate the transfected tissue culture flask for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.

**Day 2: Infection of transfected cells with rVSV-ΔG/G\*-luciferase**

9. In a 15 ml polypropylene sterile tube prepare the virus inoculation mixture by adding 5 ml DMEM-5 containing an amount of rVSV-ΔG/G\*luciferase equivalent to multiplicity of infection (MOI) of 4 using the working stock virus form Kerafast.

*Thaw the virus stock on ice before preparing the inoculum. It is recommended to generate additional working stocks by amplifying an aliquot of the rVSV-ΔG/G\*-luciferase stock form Kerafast in BHK-21/WI-2 cells transfected with pCAGGS-G (expression plasmid encoding VSV-G protein). For rVSV-ΔG/G\*-luciferase titration, viral plaque assay can be used as previously described (Whitt, 2010).*

10. Take out the transfected BHK-21/WI-2 cells in the T175 flask from Day 1 experiment from the incubator and remove the growth medium.
11. Infect the cells with the 5 ml media containing the rVSV-ΔG/G\*luciferase and make sure to distribute equally over the cells monolayer.
12. Incubate the cells for 1 hr at 37°C in 5% CO<sub>2</sub> humidified incubator and distribute the virus by gently rocking the T175 flask every 10 min.
13. During the incubation time, dilute rabbit polyclonal anti VSV-G antibodies 1:1000 dilution in a 50 ml polypropylene sterile conical tube containing 15 ml of pre-warmed DMEM-5 medium.

*Alternatively, 1 μg/ml of Anti VSV-G antibody (Kerafast Cat # EB0010) can be used. Anti-VSV-G antibody is used to neutralize excess amount of the rVSV-ΔG/G\*-luciferase that do not infect the cells. Skip this step in case of generating rVSV-ΔG/G\*-luciferase stock. Additionally, this step could be skipped if no background from residual rVSV-ΔG/G\*-luciferase is observed, however, background activity was observed in our hands (Figure 2).*

14. After 1 hr incubation, remove the virus inoculum and wash the cells twice with 12 ml of pre-warmed 1x PBS.

*Washing with 1x PBS helps to remove the rest of the rVSV-ΔG/G\*-luciferase that do not enter the cells.*

15. Add the prepared 15 ml DMEM-5 medium supplemented with anti VSV-G antibodies to the cells monolayer.

*Avoid drying the cells by working quickly between removing the growth medium and adding the medium that supplemented with the antibodies.*

16. Incubate the flask for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.

### **Day 3: Collection and storage of the generated VSV pseudoviruses**

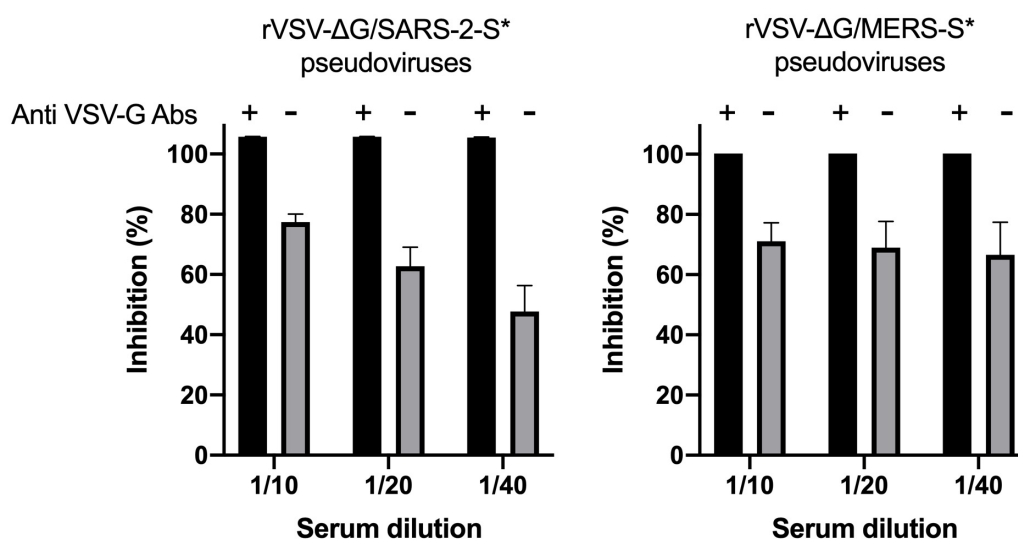
17. Next day, collect the supernatant that contains the VSV pseudoviruses in a 50 ml polypropylene sterile conical tube.

18. Remove the cells debris by centrifugation the supernatant at 600 x g for 5 min.

19. Aliquot the clarified supernatant as 1 ml into appropriately labelled sterile microcentrifuge tubes.

20. Store the generated pseudoviruses stocks at -80°C.

*Avoid thawing and freezing the virus stocks as this will affect virus titer.*



**Figure 2. Neutralization of rVSV pseudovirus generated in the absence or presence of anti VSV-G polyclonal antibodies.** Both rVSV-ΔG/MERS-S\*-luciferase and rVSV-ΔG/SARS-2-S\*-luciferase were generated in the absence or presence of anti VSV-G polyclonal antibodies and used in neutralization assay using seropositive serum samples. Using anti VSV-G polyclonal antibodies always resulted in pseudoviruses without residual rVSV-ΔG/G\*-luciferase as shown by the complete inhibition of the luciferase activity compared to the partial inhibition when pseudoviruses were generated in absence of anti VSV-G polyclonal antibodies indicated activities from residual rVSV-ΔG/G\*-luciferase.

## Basic protocol 2

### Titration assay of the generated VSV pseudoviruses by measuring luciferase activity

This protocol is based on the use of luciferase activity as a main readout of the system to titrate the produced rVSV pseudoviruses. The measured luciferase activity is defined as relative luminescence unit (RLU). This protocol is summarized in Figure 3. Different luciferase assay systems or in house made luciferase substrate and buffer could be used.

### Materials

African Green monkey kidney-derived Vero E6 cell line (ATCC catalogue # 1586)

DMEM-5 cells growth medium (see recipe)

1x PBS (Biosera catalogue # LM-S2041/1000 or equivalent)

1x trypsin (Gibco catalogue # 12484-028 or equivalent)

Supernatant contains the generated pseudoviruses *rVSV-ΔG/S\*-luciferase* (from this study)

5x Cell culture lysis reagent (CCLR) (Promega catalogue # E1531)

Luciferase assay system (Promega catalogue # E1501)

BSL-2 cabinet

Cell counter hemocytometer or equivalent

96-well cell culture plate (white or black plate with clear bottom, COSTAR or equivalent)

U shape 96-well cell culture plate (SPL life science catalogue # 30096 or equivalent)

Sterile reservoirs

37°C incubator with CO<sub>2</sub> (Heal Force HF90 or equivalent)

Water bath (Lab Tech LWB-111D or equivalent)

Polypropylene sterile conical tubes:

15-ml (Falcon catalogue # 352099, or equivalent)

50-ml (Falcon catalogue # 352098, or equivalent)

Sterile serological pipettes

5-ml (SPL life sciences catalogue # 91005 or equivalent)

10-ml (SPL life sciences catalogue # 91010 or equivalent)

25-ml (SPL life sciences catalogue # 91025 or equivalent)

Micropipettes

Micropipette tips

10 µl filter tips, low retention (BIOLOGIX catalogue # 23-0011 or equivalent)

200 µl filter tips, low retention (BIOLOGIX catalogue # 23-0201 or equivalent)

1000 µl filter tips, low retention (BIOLOGIX catalogue # 23-1001 or equivalent)

Inverted microscope (Olympus CK30 or equivalent)

Timer

Luminometer (BioTek Synergy 2 microplate reader or equivalent).

70% Ethanol; to sanitize all materials before use it inside the BSL-2 cabinet.

### ***Day 1: Cell preparation***

1. Count Vero E6 cells from confluent T75 tissue culture flask using a hemocytometer slide and trypan blue solution. See Current Protocols article: Phelan & May (2015) for cells counting and basic cell culture techniques.

*It is preferred to subculture confluent Vero E6 at 1:4 ratio 48 hr before use. Huh-7 cells could be used instead.*

2. Prepare 11 ml of Vero E6 cells suspension at a density of  $2 \times 10^5$  cells/ml in a 50 ml polypropylene sterile conical tube.

*Pre-warmed DMEM-5 is used to prepare the cells suspension.*

3. Seed the Vero E6 cells suspension in a 96-well white or black cell culture plate with clear bottom by distributing 100 µl of the cells/well using multichannel pipette, sterile filtered tips and sterile reservoir.

*Every 100 µl should contain  $2 \times 10^4$  cells in total.*

4. Incubate the seeded 96-well plate for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.

*To save time, steps 1-4 can be done in the last day of Basic Protocol 1 after collecting the pseudovirus.*

### ***Day 2: Cell infection with generated VSV pseudoviruses bearing CoV S gene***

5. In a sterile U shape 96-well cell culture plate, add 60  $\mu$ l of pre-warmed DMEM-5 to all wells in column 12 as a negative cell control; cell only control (CC).
6. Add 60  $\mu$ l of pre-warmed DMEM-5 to all wells in columns 1 to 11 in rows B to H using multichannel pipette, filtered tips and sterile reservoir.
7. Thaw the supernatant containing generated VSV pseudotyped viruses on ice.
8. Add 120  $\mu$ l of the supernatant containing rVSV- $\Delta$ G/S\*-luciferase pseudoviruses in wells A1 to A8.  
*As an example, rVSV- $\Delta$ G/MERS-S\*-luciferase pseudoviruses can be added in wells A1 to A4 and rVSV- $\Delta$ G/SARS-2-S\*-luciferase pseudoviruses in wells A5 to A8.*
9. As positive control (PC), add 120  $\mu$ l of the supernatant containing rVSV- $\Delta$ G/G\*-luciferase pseudoviruses in wells A9 to A11.
10. Remove 60  $\mu$ l from virus-containing wells in row A (A1-A11) and perform 1:2 serial dilution downward to all wells below using multichannel pipette and filtered tips.  
*Other dilutions such as 1:3 or 0.5 log could be used.*
11. During each dilution step, mix well by pipetting eight times up and down.
12. Continue the dilution until row H and discard the final 60  $\mu$ l from the last wells in row H.
13. Remove the media from the plated Vero E6 cells in 96-well plate that was seeded on Day 1.
14. Using multichannel pipette and filtered tips, transfer 50  $\mu$ l from all wells in the U shape 96-well cell culture plate to corresponding wells in the 96-well plate of Vero E6 cells.
15. Add 50  $\mu$ l of pre-warmed DMEM-5 to all wells in 96-well plate of Vero E6 cells
16. Incubate the plate for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.

### ***Day 3: Luciferase assay***

17. Prepare 1x lysis buffer from 5x CCLR in a 15 ml polypropylene sterile conical tube by adding 4 volumes of water to 1 volume of 5x CCLR. A total of 2.5 ml of 1x lysis buffer will be enough for each 96-well plate (20  $\mu$ l/ well).

*Equilibrate 5x CCLR to room temperature before preparing the 1x lysis buffer.*

18. By using luciferase assay system, prepare the luciferase assay reagent by adding 10 ml luciferase assay buffer to a vial containing lyophilized luciferase assay substrate.

*Equilibrate luciferase assay buffer to room temperature before preparing the reagent. Avoid exposure of the luciferase assay reagent to multiple freeze-thaw cycles.*

19. Remove the growth media from all wells in the 96-well cell culture plate of Vero E6 cells from Day 2.

20. Rinse the cells in all well with 50  $\mu$ l 1xPBS and make sure not to dislodge the cells.

*Ensure complete removing of any residual liquid.*

21. Add 20  $\mu$ l of the prepared 1x cells lysis buffer to each well.

22. Add 50  $\mu$ l of the prepared luciferase reagent to each well.

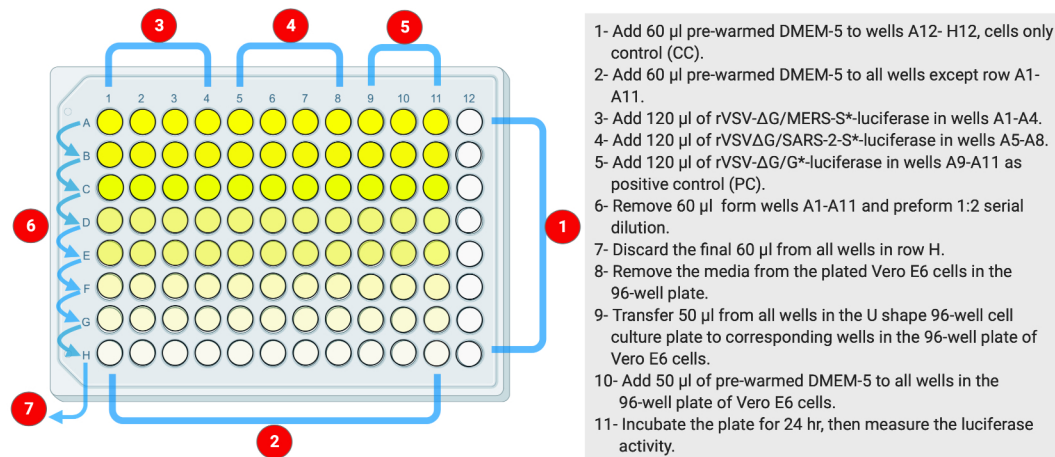
*Work with two columns each time to complete the steps 21-23, which include cell lysis, addition of luciferase reagent and measuring the luciferase activity. Repeat cycle for the remaining columns. Alternatively, a luminometer supplied with two injectors for both lysis buffer and luciferase substrate can be used to facilitate the process by measuring light produced from the reaction  $\sim$ 8 sec after adding the substrate using an integration time of 5–30 sec.*

23. Measure the light produced for a period of  $\sim$ 8 sec using luminometer and save the results

*The reaction is nearly constant for about 1 min and then decays slowly, with a half-life of approximately 10 min. The typical delay time is 2 sec, and the typical read time is 10 sec.*

24. Plot virus dilution vs. RLU readout to select the needed amount of virus for neutralization assay. Select a dilution that result in a signal above cell-only control and in the linear part of the curve.

*We use a dilution of the generated pseudovirus that yields  $5 \times 10^4$  RLU.*



**Figure 3. The layout of U shape 96-well cell culture plat for rVSV-ΔG/S\*-luciferase pseudovirus titration in Basic protocol 2.** The preparation steps are indicated in sequential numbers. Created with BioRender.com.

### Basic protocol 3

#### Neutralization assay to determine CoVs specific nAbs titers in serum samples

As in Basic protocol 2, measuring of nAbs titers depends on using luciferase-based assay. Inhibition of the generated pseudovirus entry into Vero E6 cells by CoV antibodies is correlated with the decreased levels of luciferase expression signals. This assay could be used to measure nAbs titers from different species including humans and animals as well as testing monoclonal antibodies. Figure 4 illustrates the workflow.

#### Materials

All materials as in Basic Protocol 2

Serum samples

#### Day 1: Cell preparation

1. Seed  $2 \times 10^4$  Vero E6 cells per well in a 96-well white or black cell culture plate with clear bottom. This can be achieved by following steps 1-4 in Basic Protocol 2.

#### Day 2: Pseudovirus neutralization assay

2. In a sterile U shape 96-well cell culture plate, add 60 μl of pre-warmed DMEM-5 to all wells in columns 1 to 12 in rows B to G.

3. Add 120  $\mu$ l of pre-warmed DMEM-5 to wells H1 to H6 to serve as negative cell control; cell only control (CC).
4. Add 60  $\mu$ l of pre-warmed DMEM-5 to wells H7 to H12 to be used as virus control (VC).
5. Add 120  $\mu$ l of 1:10 dilution of heat-inactivated serum samples in wells in row A, add each sample in duplicate.

*Heat-inactivate serum samples at 56°C for 30 min.*

6. Remove 60  $\mu$ l from serum-containing wells in wells A1-A12 and perform 1:2 serial dilutions downward to all wells below.

*Other dilutions such as 1:3 or 0.5 log could be used.*

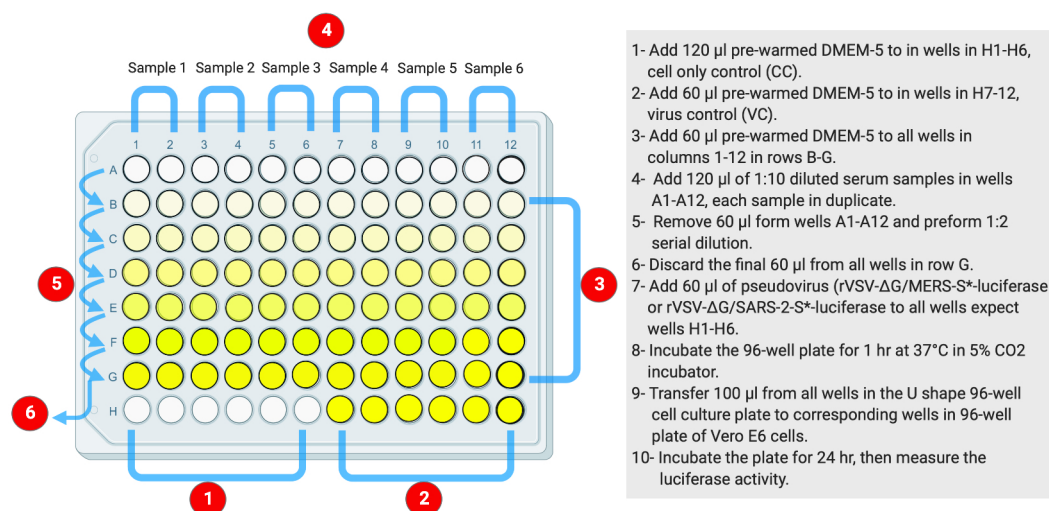
7. During each dilution step mix well by pipetting eight times up and down.
8. Continue the dilution until row G and discard the final 60  $\mu$ l from the last wells in row G.
9. Prepare rVSV pseudovirus suspension at a concentration of  $1 \times 10^6$  RLU per ml.

*A total of 7.5 ml are needed for one 96-well plate*

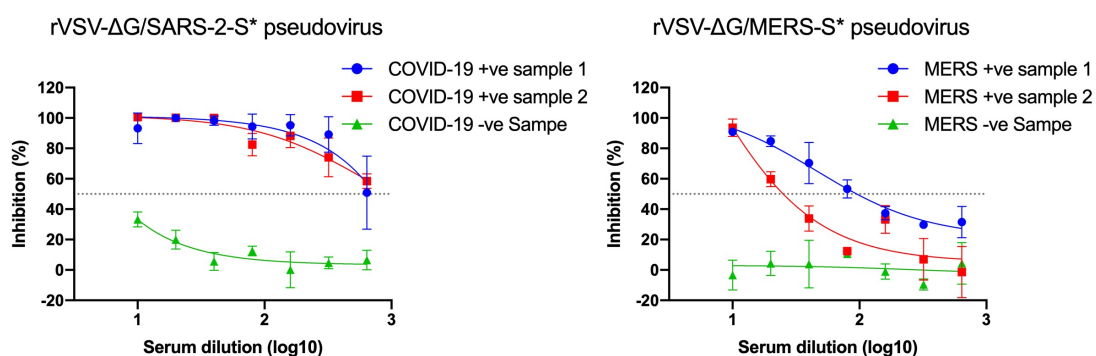
10. Add 60  $\mu$ l of pseudovirus suspension into each well in the plate except wells H1 to H6 (CC).
11. Incubate the plate for 1 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.
12. Take out the plated Vero E6 cells in 96-well cell culture plate from the incubator that was seeded on Day 1 and remove the growth medium.
13. Using a multichannel pipette and filtered tips, transfer 100  $\mu$ l from all wells in the U shape 96-well cell culture plate to corresponding wells in the 96-well plate of Vero E6 cells.
14. Incubate the plate for 24 hr at 37°C in 5% CO<sub>2</sub> humidified incubator.

### ***Day 3: Luciferase assay***

15. Follow the same 17-23 steps that have been described in the luciferase assay in Basic Protocol 2 to measure luciferase activity. An example of neutralization assay results is shown in Figure 5.



**Figure 4.** The layout of U shape 96-well cell culture plat for rVSV-ΔG/S\*-luciferase pseudovirus neutralization assay in Basic protocol 3. The preparation steps are indicated in sequential numbers. Created with BioRender.com.



**Figure 5.** Example of neutralization activity using the generated pseudoviruses. Inhibition of rVSV-ΔG/MERS-S\*-luciferase and rVSV-ΔG/SARS-2-S\*-luciferase pseudoviruses using seropositive and seronegative human serum samples. Data were plotted using four-parameter inhibition curves. Serum samples were diluted starting from 1:10 to 1:640.

## Reagents and Solutions Recipe

### DMEM-5 (1L)

In 1L beaker add:

500 ml distilled water

13.5 g DMEM powder (Biosera catalogue #PM-D1114/10L or equivalent)

Dissolve using magnetic stirrer

Add distilled water up to 890 L

Filter the prepared medium using a 0.22- $\mu$ m Stericup vacuum filtration system  
Inside BSL-2 cabinet, add the following sterile components to the prepared mixture:

20 ml of 7.5% Sodium bicarbonate (Gibco catalogue # 25080-060 or equivalent)

10 ml of 100x Penicillin/Streptomycin (Biosera catalogue # XC-A4122/100 or equivalent)

10 ml of 200 mM L-Glutamine (Biosera catalogue # XC-T1715/100 or equivalent)

10 ml of 1 M HEPES buffer (Biosera catalogue # LM-S2030/500 or equivalent)

10 ml 100x MEM non-essential amino acids (Biosera catalogue # XC-E1154/500 or equivalent)

50 ml FBS (Biosera catalogue # FB-1001H/500 or equivalent)

Store at 4°C for a month.

## COMMENTARY

### Background Information

The detailed protocols described here can serve as convenient methods to detect MERS-CoV and SARS-CoV-2 nAbs in serum samples under BSL-2 conditions. The pseudovirus assays detailed here could also be used to evaluate the immunogenicity of vaccines and potency of monoclonal antibodies, other biologics and small molecules. These assays rely on a well-established technique using rVSV- $\Delta$ G/G\*-luciferase pseudovirus system. Most reagents required for this system are commercially available and could be adopted and used by researchers and laboratories around the world. Such assay provides a number of advantages over standard serological assays including ability to test for nAbs in serum samples under BSL-2 conditions with minimal equipment and relatively low cost.

### Critical Parameters Troubleshooting

#### *Low/ poor transfection efficiency*

A number of important parameters should be considered to increase the transfection efficiency including:

*BHK-21/WI-2 cells:* Low transfection efficiency could lead to low pseudovirus titers. Using healthy and low passage BHK-21/WI-2 cells at ~70% confluency enhances the experiment outcomes. Starting with low or large number of cells could lead to reduction in the uptake of foreign DNA.

*Plasmid DNA:* It is highly recommended to use high-quality expression DNA plasmid encoding the gene of interest for cells transfection. A number of commercial kits can be used to obtain purified plasmid free of RNA, nucleases, proteins, chemicals and microbial contamination.

*Transfection reagent:* For preparing the transfection mixture, it is required to use serum-free cell culture medium such as Opti-MEM<sup>®</sup> I (1x) reduced-serum medium. We used Lipofectamine<sup>™</sup> 2000 transfection reagent and it worked well in our hands, however, other reagents or methods could be used.

### ***Luciferase assay background readings***

Some steps need to be duly done to avoid the luciferase reading errors, which are:

**Removal of rVSV-ΔG/G\*-luciferase pseudovirus after infection:** One of the most common problems that could occur during the generation of pseudoviruses (Basic Protocol 1) is having residual or background rVSV-ΔG/G\*-luciferase pseudovirus in the collected supernatant (Figure 2). Complete removal of excess rVSV-ΔG/G\*-luciferase that do not infect the cells could be done by washing the cell monolayer twice with 1x PBS as well as using DMEM-5 supplemented with anti VSV-G antibodies.

**Dilution of pseudovirus and serum samples:** Changing pipette tips between steps (Basic Protocols 2 and 3) is important to get accurate luciferase activity results. Additionally, complete removal of residual liquid after washing wells and before adding the luciferase reagent helps reducing the background noise.

### **Understanding Results**

Measuring nAbs titers using pseudoviruses depends on luminescence signal reads obtained from the activity of the expressed luciferase. The recommended amount of VSV pseudovirus to be used in neutralization assays should have a signal above cell-only control and in the linear part of the curve. It is recommended to use a dilution of the pseudovirus that yields  $5 \times 10^4$  RLU. The nAb titers (NT<sub>50</sub>) in serum samples could be calculate as the reciprocal of the serum dilution that reduced luciferase activity by

50%. The inhibition rate is calculated as  $100 - [(mean\ RLU\ from\ each\ sample - mean\ RLU\ from\ CC) / (mean\ RLU\ from\ VC - mean\ RLU\ from\ CC) \times 100]$ .

### **Time Considerations**

Basic Protocols 1 and 2 can be completed in about 7 consecutive days. However, it is possible to do it in 6 days if seeding of Vero E6 cells in 96-well plates for titration is prepared in the same day of collecting the pseudovirus supernatant. In details, it takes 4 days to produce VSV pseudoviruses bearing CoV S protein (Basic Protocol 1). Once done successfully, the produced pseudoviruses can be stored at  $-80^{\circ}\text{C}$  for long time. Titration of generated pseudovirus (Basic Protocol 2) is usually done in 3 days. Pseudovirus neutralization assay (Basic Protocol 3) takes 3 days, and up to 6 serum samples could be tested per plate.

### **Acknowledgements**

We also would like to thank King Fahd Medical Research Center and King Abdulaziz University (KAU) for their continuous support. The authors gratefully acknowledge Kerafast for providing materials to conduct the work.

### **Funding**

The authors extend their appreciation to the deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number (436).

### **Conflicts of Interest**

None

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