

Review Article



SARS-CoV-2 Antibody Neutralization Assay Platforms Based on Epitopes Sources: Live Virus, Pseudovirus, and Recombinant S Glycoprotein RBD

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

ACE2, angiotensin converting enzyme-2;
COVID-19, coronavirus disease-2019; CP,

ABSTRACT

The high virulent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus that emerged in China at the end of 2019 has generated novel coronavirus disease, coronavirus disease 2019 (COVID-19), causing a pandemic worldwide. Every country has made great efforts to struggle against SARS-CoV-2 infection, including massive vaccination, immunological patients' surveillance, and the utilization of convalescence plasma for COVID-19 therapy. These efforts are associated with the attempts to increase the titers of SARS-CoV-2 neutralizing Abs (nAbs) generated either after infection or vaccination that represent the body's immune status. As there is no standard therapy for COVID-19 yet, virus eradication will mainly depend on these nAbs contents in the body. Therefore, serological nAbs neutralization assays become a requirement for researchers and clinicians to measure nAbs titers. Different platforms have been developed to evaluate nAbs titers utilizing various epitopes sources, including neutralization assays based on the live virus, pseudovirus, and neutralization assays utilizing recombinant SARS-CoV-2 S glycoprotein receptor binding site, receptor-binding domain. As a standard neutralization assay, the plaque reduction neutralization test (PRNT) requires isolation and propagation of live pathogenic SARS-CoV-2 virus conducted in a BSL-3 containment. Hence, other surrogate neutralization assays relevant to the PRNT play important alternatives that offer better safety besides facilitating high throughput analyses. This review discusses the current neutralization assay platforms used to evaluate nAbs, their techniques, advantages, and limitations.

Keywords: Neutralizing antibody; Neutralization assay; Pseudovirus; S glycoprotein; SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) was first reported in December 2019 in Wuhan, China (1). Until the end of September 2021, around 233 million people have been infected, causing 4.7 million deaths (2). This infectious disease is characterized by shortness of breath which is associated with pneumonia-like symptoms, adding the new line to the prior infectious severe respiratory disease list after severe acute respiratory syndrome (SARS) and Middle

convalescence plasma; CPE, cytopathic effects; ER, endoplasmic reticulum; GFP, green fluorescence protein; GRNT, GFP reduction neutralization test; HIV, human immunodeficiency virus; MERS, Middle East respiratory syndrome; nAbs, neutralizing Abs; PRNT, plaque reduction neutralization test; RBD, receptor binding domain; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; VSV, vesicular stomatitis virus.

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East respiratory syndrome (MERS) (3). SARS caused by SARS coronavirus (SARS-CoV) also first appeared in China in 2002, while MERS caused by MERS-CoV appeared in 2012, starting from the Middle East (4). Furthermore, an electron microscope image reveals that COVID-19 is also caused by a coronavirus later named SARS-CoV-2 by WHO, which announced it on February 12, 2020 (5-7). Walls et al. (8) reported that the coronavirus-specific morphology of SARS-CoV-2 with virus particle sizes ranging from 70 to 90 nm was observed under a wide variety of intracellular organelles, most specifically in vesicles.

SARS-CoV, MERS-CoV, and SARS-CoV-2 all belong to the Coronaviridae family, subfamily of Orthocoronavirinae subfamily and genus of the β -coronavirus (9). As a β -coronavirus, SARS-CoV-2 harbors a positive single-stranded RNA genome of about 30 kb as its material genetic, which encodes about 27 structural and non-structural (nsps) proteins (9,10). Genomic sequencing analyses of SARS-CoV-2 viruses isolated from 3 pneumonia patients in Wuhan, China (BetaCoV/Wuhan/IVDC-HB-01/2019, accession ID: EPI_ISL_402119; BetaCoV/Wuhan/IVDC-HB-04/2020, accession ID: EPI_ISL_402120; BetaCoV/Wuhan/IVDC-HB-05/2019, accession ID: EPI_ISL_402121) reveal 86.9% similarity of the virus genome to SARS-like CoV genome originated from bats (bat-SL-CoVZC45, MG772933.1) (11). The high sequence similarity between SARS-CoV-2 and SARS-CoV allows the structural similarity of these 2 types of viruses (12,13).

The SARS-CoV-2 Orf1ab and Orf1a genes located in 5'-terminal encode the pp1ab and pp1a polyprotein replication which consist of about 15 nsps that produce papain-like cysteine protease (Plpro), RNA-dependent RNA polymerase (RdRp), and helicase. Meanwhile, the 3'-terminal of the viral genome encodes the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (1,9,14). The nucleocapsids are assembled in the endoplasmic reticulum (ER), followed by budding into smooth vesicles derived from the Golgi apparatus (15,16). Then, the membrane-derived lipid bilayer embedded with the spike, membrane, and coronavirus envelope encapsulates the helical nucleocapsid comprising the viral RNA (17). The proposed mechanism for the export of viruses to the extracellular space is via fusion of the transport compartment membrane with the cell plasma membrane (18), and translocation to the plasma membrane is required to release the viral particles by a fusion mechanism (19).

The life cycle of SARS-CoV-2 might be similar to that of SARS-CoV. Zhao et al. (7) reported that SARS-CoV-2 enters cells through membrane fusion and matures in vesicles based on TEM data. This SARS-CoV-2 entrance mechanism could be described in more detail since the structure of the SARS-CoV-2 spike has been resolved (20). The S glycoprotein of SARS-CoV-2 contains S1 and S2 subunits. The S1 subunit facilitates the virus attachment to the angiotensin-converting enzyme-2 (ACE2) receptor, the same receptor that mediates SARS-CoV infection (21). In addition, the S2 subunit mediates fusion with the membrane of the host cell (9). The S1 subunit harbors the receptor-binding domain (RBD), which shows affinity to the human ACE2 in which the S1 RBD-ACE2 bond will trigger conformational changes in the S2 subunit, allowing the fusion process of the viral molecule with the host cell (14), thereby making S glycoprotein as an essential target for vaccine development (8). However, in contrast to SARS-CoV, SARS-CoV-2 S protein possesses a furin-like protease cleavage site as a target for transmembrane serine protease 2 (TMPRSS2) (22), that does not present in the SARS-CoV, which is supposed to contribute to its high transmissibility across humans (20,23). In addition, SARS-CoV-2 also generated more elevated levels of intracellular RNA that may facilitate a faster replication rate (24).

Less than a year of a relative evolutionary stasis after the SARS-CoV-2 appearance, there had been no report of its emerging mutants (25,26). However, SARS-CoV-2 virus can evolve to adapt to the challenging nature. At the end of 2020, new SARS-CoV-2 mutants in the perspective of “variants of concern” have been reported impacting its characteristics, transmissibility, infection severity, and antigenicity. The virus has undergone genetic changes to prevent attachment to neutralizing Abs (nAbs), escaping from the body’s immune system (26). Nevertheless, how the mutations affect the antigenic phenotype of SARS-CoV-2 allows the variants to evade immunity conferred by natural infection or vaccination remains to be determined (26). In addition, the mutation might occur to increase the virus affinity to its receptor (27). Surveillance has revealed that variants harboring mutations in SARS-CoV-2 spike are circulating and affect immune recognition to the degree that requires immediate attention. SARS-CoV-2 variants of concern as of September 16, 2021, are Beta, Gamma, and Delta. In these variants, strong evidence indicates a significant impact on transmissibility, severity, and immunity (28). To learn more about vaccine efficacy and the consequences of an antigenic variation on vaccine effectiveness, information about vaccine status and types of variants from individuals infected with SARS-CoV-2 is crucial.

Since neither medicine nor vaccine was successfully developed for SARS-CoV treatment, the searches for SARS-CoV-2 medication and vaccine remained elusive at the beginning of the pandemic (29,30). Nowadays, while several types of COVID-19 vaccines are available in the market, clinical treatment still demands effective medicines (31). Therefore, convalescence plasma (CP) that contains nAbs is still promising to be used in treating COVID-19 patients (32,33). These nAbs are capable of blocking SARS-CoV-2 interaction with hACE2 through the RBD, preventing virus entry (34). Recently, SARS-CoV-2 cases are still increasing globally, including in tropical countries (35). People contracted the virus show various symptoms ranging from mild to severe, including fever, cough, muscle pain, and respiratory problems despite the population of asymptomatic people. COVID-19 showed dynamic changes in pathogenesis along with the appearance of new SARS-CoV-2 mutants with distinct viral infectivity (22). At the same time, the treatment of COVID-19 remains based on the handling of the symptoms (36). Virus elimination occurs depending on the body immunity, which involves the generation of nAbs. Among COVID-19 treatments, CP containing nAbs transfusion has shown promising clinical outcomes, ranging from reducing viral load to accelerating hospital discharge (22,32). Attempts to isolate neutralizing monoclonal Abs from single B cells of COVID-19 patients are also conducted to provide purified nAbs for COVID-19 therapy (37). Furthermore, vaccination programs are massively carried out to reach herd immunity by igniting the nAbs production to defend against the virus (38). Hence, nAbs play crucial roles in patients’ immunity to accelerate their recovery from SARS-CoV-2 infection.

The evaluation of Ab titers is a critical requirement to assure a sufficient level of anti-SARS-CoV-2 Abs, especially the nAbs present in the plasma (39,40). The titer of nAbs in CP is associated with the quality of the CP to be used for transfusion (41). On the other hand, the titer of nAbs in the plasma of post-vaccinated patients determines the vaccine efficacy (42). Thus, assessments of serological nAbs are significantly needed in the health care systems to ensure the progress of clinical treatment and to determine the recommended vaccines. Several neutralization assay platforms based on the epitopes sources can be performed to measure the titers of nAbs, including SARS-CoV-2 live virus, recombinant pseudovirus, and S glycoprotein RBD sub-unit based neutralization assays. In this review, we discuss the Ab neutralization assay platforms, including assays utilizing wild-type virus, SARS-CoV-2

pseudoviruses, and surrogate virus assay utilizing recombinant spike RBD and recombinant human ACE2, their techniques, strength, and limitation.

EVALUATION OF NABs TITERS

In general, the examination of Ab titers can be categorized into infectivity assay and physicochemical assay. Viral infectivity assay is carried out according to the nature of nAbs specific to the virus to bind and prevent it from interacting with its target receptor, known as neutralization assay (43). More recently, virus pseudotyped has been developed to replace the use of original highly pathogenic viruses (44). On the other hand, the physicochemical evaluation of nAbs involved ELISA, which is based on the Ag-Ab interaction, especially with the use of specific receptor binding sites (45).

Virus based neutralization assay

Neutralization assay using the original live viruses describes the blockage of nAbs-receptor binding, cell entry, and virus replication within the host cells (43,46). Thus, using this method, we can investigate nAbs activities to block virus-receptor attachment implicated in reducing virus infectivity and cytotoxicity, representing the actual events *in vivo*. In the absence of Abs, the viruses will infect the cells causing the cells to lyse, which appeared as plaques during observation. Moreover, the addition of the nAbs will reduce the plaque count. Viral plaque assay represents the *in vitro* cell infection model and becomes the standard protocol in immunology, viral pathology, and vaccine development (47,48). However, to perform this kind of assay, we have to conduct virus isolation and propagation, which must be carried out in the designated facility associated with virus pathogenicity. Due to its virulent nature, the SARS-CoV-2 virus is classified as a virus that needs a BSL-3 facility (49). However, the number of BSL-3 facilities is generally limited due to its demands of negative pressure, tight containment, experienced personnel, and strict laboratory management (50). Thus, the number of laboratories capable of conducting SARS-CoV-2 virus-based neutralization assay is also limited (51).

SARS-CoV-2 targets specific cells and shows distinct tropism in different cell lines that affect different levels of observable cytopathic effects (CPE) (52,53). In humans, the tropism among organs is also different in which lung elicits the most significant impact of virus infection. Lung carcinoma cells Calu-3, especially the polarized cells, exhibit virus production observed by RT-PCR and generate CPE after infection with SARS-CoV-2. Along with Calu-3, colon cancer cells Caco-2 also facilitate virus replication even though these cells do not develop the same degree of cytotoxicity. In addition, compared to Caco-2, kidney cells 293FT, and liver cells Huh7, Calu-3 shows the highest abundance of viral proteins evaluated by proteomic analyses. Huh7 and 293FT express endogenous TMPRSS2 but do not express ACE2 at observable points by western blot (53). On the other hand, besides ACE2 expression, Calu-3 and Caco-2 are enhanced with endogenous TMPRSS2 expression that plays a vital role in promoting membrane fusion (53,54).

As SARS-CoV-2 infects human cells via human ACE2, the virus is also capable of infecting animal cells. Among animal cells, African green monkey epithelial cells that have been widely used in virology, Vero E6, show the highest CPE while playing a role as a host for virus propagation, making them as most important cells for neutralization assay (37). Interestingly, while replicating within Vero cells, the SARS-CoV-2 viruses also evolve and generate new

mutants to promote higher infectivity rates (24). In comparison to human kidney cells proximal tubular epithelial cells, glomerular mesangial cells, and glomerular epithelial cells (podocytes), Vero E6 cells show the highest endogenous surface ACE2 expression analyzed by flow cytometry that exhibits about 76% of the cell population expressing this receptor (55). In addition, the ACE2 expression level shown by western blot data is slightly lower than Calu-3, with a similar degree of virus susceptibility and cytotoxicity (53).

Plaque reduction neutralization test (PRNT)

Vero E6 cells maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/100 µg/ml streptomycin in humidified CO₂ incubator can be readily infected by SARS-CoV-2 virus (56). The infecting virus replicates within the cells causing the cell to be lysed and form visible plaques, which count as plaque-forming units per ml (PFU/ml). Thus, the PFU/ml corresponds to the virus infectivity level which is determined through titer evaluation. In virus-based neutralization assay using Vero E6 cells, briefly, the cells are seeded in a multiwell-plate then infected with the virus in the following day. The inoculum is removed after 1-h incubation, and the infected cells are overlaid with a semi-solid medium containing agar or carboxymethyl cellulose to prevent further sporadic infection. The inactivated CP or serum isolated from either the COVID-19 patients or vaccinated patients are mixed with virus solution corresponding to the designated PFU/ml. If the serum added to the culture contains nAbs, it will inhibit Vero E6 cell infection, which reduces viral PFU counted after 2–3 days incubation. The higher the Ab titer in the plasma or serum sample, the lower PFU will be observed (56,57). Therefore, virus neutralization assay is also known as PRNT as well. To ease the plaque investigation, they can be stained by using a dye such as crystal violet. In addition to plaque visualization by crystal violet staining, fluorescence-based staining also can be carried out to observe the infected cells by utilization of fluorochrome-tagged secondary Abs (58). Basically, the number of cells that expressed fluorescent signals is defined as the number of infected cells (59).

PRNT, which is carried out in Vero cells, is a crucial part of evaluating vaccine efficacy, COVID-19 patients' immunity, and the quality of CP. There are reports on the application of PRNT on CP evaluation designated for COVID-19 patient treatment. Shen et al. (60) had performed a serum neutralization assay of SARS-CoV-2 virus by nAbs of CP in Vero cells. Technically, the serum samples need to be heated at 56°C for 30 minutes to inactivate the life viruses. Serial dilution of serum is made to be incubated with the viruses to determine the nAbs titer. The mixture is then added to the confluent monolayer Vero cells in a 96-well plate in which only free viruses are able to attach ACE2 receptors. The visualized plaques are then observed by using a microscope to determine the neutralizing Ab titer.

Shen et al. (60) reported that by this test, the CP neutralizing Ab titers fall between 80 and 480. PRNT was also performed by Duan et al. (61) to determine the neutralizing activity of CP. This test was conducted in a high biosafety level (BSL)-4 laboratory of Wuhan Institute of Virology, China toward CPs collected from 40 recovered patients who were recommended as plasma donors. As the results, 39 patients showed high Ab titers at least 1:160 (61). Nevertheless, PNRT also possesses several disadvantages as its implementation requires virulent virus propagation in a BSL-3 facility. In addition, it does not allow rapid screening of plasma to meet the current high demand. In accordance with this matter, PNRT for CP is recommended but not obliged by FDA. The retained plasma samples can be tested later after the administration of CP (62).

Pseudovirus based neutralization assay

A SARS-CoV-2 virus consists of a coronavirus backbone and virus envelope containing spike S glycoprotein (63). Current reverse genetic technology enables the synthesis of pseudovirus, a recombinant viral particle with its backbone and envelope proteins derived from different viruses (64,65). The virus backbone used for pseudotyping is generally derived from less pathogenic viruses such as vesicular stomatitis virus (VSV) (66). VSV is an animal virus that can infect most mammalian cells (67). This virus has been widely used as the core for SARS-CoV-2 pseudotyping which can be integrated with the reporter proteins (Table 1) (48,54,68,69,70). The genetic material of the host-virus has been modified so that they are unable to produce their own surface protein, replaced with the spike glycoprotein of the designated virus. Thus, in developing the SARS-CoV-2 pseudovirus, the S glycoprotein is engineered to be incorporated into other manipulated virus backbones (44). As a result, the generated recombinant pseudotyped virus will bind the SARS-CoV-2 receptor, hACE2, instead of interacting with the host-virus receptors.

SARS-CoV-2 pseudotyping techniques

Pseudovirus can be engineered to be either replication-competent or incompetent depending on the modification of the virus genome (47,48,68,69). Compared to wild-type viruses, replication-incompetent pseudoviruses can only undergo a single infection cycle in the infected host cells. Hence, most SARS-CoV-2 pseudoviruses are engineered to form replication-incompetent ones to essentially devoid of virulent viral components and generate more safety to be handled in BSL-2 laboratories (48).

In a replication-competent pseudovirus, the wild-type spike gene is substituted with the gene encoding the designated spike gene in order the virus genome bears the complete material genetic to produce infectious recombinant pseudovirus. For instance, to produce a replication-competent SARS-CoV-2 pseudotype using VSV backbone, we need to generate a VSV genome bearing knocked out G glycoprotein gene to enable the incorporation of S glycoprotein into the VSV backbone (68). The procedure to generate pseudotyped viruses is less complicated than replication-incompetent pseudovirus because the G glycoprotein gene is directly substituted with S glycoprotein (rVSVΔG/SARS-CoV-2 S*), so the additional plasmid is not needed to complement the removal of the G-glycoprotein gene. Supporting plasmids encoding viral structural proteins: nucleoprotein (N), phosphoprotein (P), and large polymerase protein (L) are also added to increase packaging efficiency, which

Table 1. SARS-CoV-2 pseudotyping platforms

Virus backbone	Reporter	Ref	Packaging cells	Replication competent	Plasmids
VSV	GFP	30	293T	Yes	plasmid for expression of rVSV backbone, N, P, M, and L of VSV, and T7 polymerase
	Luciferase, GFP, DsRed	31	293T	No	pcDNA3.1.S2; pVSV-*G-GFP; pVSV-G-DsRed; pVSV-L-GFP; pBS-P-T; pBS-L-T; pBS-G; pC-VSV-G
	Luciferase	39	293T	No	pCGI-SARS-CoV-2-S, VSV*DG-fLuc
	GFP	51	Vero CCL81	Yes	rVSV-eGFP-SARS-CoV-2-SAA, VSV N, P, L, G
	GFP	52	Vero E6, BHK-21, 293T	No	pCAG-nCOVS, pCAG-nCoVSDel18, VSVdG-EGFP-G
	GFP	58	293T	No	Pseudotyped rVSVΔG-GFP*G; pCAGGS-G-Kan; pCMV14-3X-Flag-SARS-CoV-2 S; psPAX2 GagPol; pMD2.GVSV-G
	Luciferase	61	BHK-21	No	Pseudotyped rVSV-DG/G* luciferase, pCAGGS-G-kan
HIV-lentivirus	Luciferase	56	Lenti-X-293T	No	pNL4-3.Luc.RE, pSRC332 (SARS-CoV-2-S subcloned into pcDNA3.1)
	Luciferase	57	293T	Yes	The HIV gag/pol, and Luciferase reporter (pHR'CMV-Luc)
	miRFP	62	293T	No	pNL43.Luc.R-E-miRFP, pCAGGS-S, pCAGGS-S-D614G, pCAGGS-S-AAAR, pCAGGS-Str; pVSV-G
	Luciferase	63, 64	293T	No	pNL4-3.luc.RE, pcDNA3.1-SARS-CoV-2-S

expression can be regulated by a T7 promoter of vaccinia virus. Therefore, to promote the expression of recombinant protein, the vaccinia virus is needed. Alternatively, the vaccinia virus also can be replaced by using plasmid, which encodes the T7 polymerase of this virus.

For generating replication-incompetent pseudovirus, the gene encoding host-virus spike protein is knocked out and complemented with an additional recombinant plasmid DNA to express the spike gene of interest (64). Several protocols can be used for this purpose, including the utilization of one set of plasmids bearing VSV- Δ G virus genome, plasmids encoding N, P, L, G, and an additional plasmid bearing S glycoprotein gene. Basically, the protocol is divided into 2 steps, first, the generation of rVSV- Δ G backbone complemented with G glycoprotein, second, pseudotyping by addition of rVSV into cells overexpressing S glycoprotein. Similar to Case et al. (68), Whitt (71) also regulates the expression of VSV genes by using the T7 promoter that needs T7 polymerase of the vaccinia virus. By this method, either propagation and purification of the vaccinia virus or plasmid bearing T7 polymerase gene are needed before generating recombinant VSV (rVSV). This T7 pol plasmid also can be cotransfected together with other plasmids to generate rVSV by reverse genetic or by generating stable cells expressing this T7 polymerase prior to transfection with VSV plasmids (Fig. 1). To ease the pseudotyping process, fortunately, rVSV lacking G glycoprotein gene in the genome can be obtained from Kerafast (rVSV- Δ G/G* Luciferase, EH1020-PM). The rVSV is ready to use for pseudotyping by adding the virus solution into cells overexpressing S glycoprotein (72). This rVSV also can be amplified by infecting the cells overexpressing G glycoprotein using this recombinant virus so that the G glycoprotein gene will complement the deleted gene in the virus genome.

Besides the utilization of VSV core, HIV lentivirus core also has been used for pseudotyping (Table 1). VSV elicits bullet-shaped conformation, while HIV lentivirus, resembling SARS-CoV-2, exhibits round-shaped conformation. Similar to VSV pseudotyped, HIV lentivirus pseudotyped also can be engineered to be replicative or non-replicative by modifying the S glycoprotein gene to substitute the HIV envelope in the virus genome or to complement it by subcloning into an additional plasmid (73-77).

Distinct packaging cell lines can be used to generate pseudoviruses including Vero, BHK-21, and 293T cells, in which Vero cells generate the highest pseudovirus titer (69). However, the problems arise in accordance with the lower pseudovirus titer when the S glycoprotein is expressed at a low level and slowly releases to the cell membrane. The S glycoprotein gene needs to be subcloned into a plasmid DNA vector that enables a high expression level of S glycoprotein to support the production of functional pseudoviruses (78). Alternatively, a viral vector can be used to generate stable cells that over-expressed the S glycoprotein (79). Next, to accelerate the release of S glycoprotein from the ER, about 18–19 amino acids at the C terminal domain associating with the ER-retention tail can be removed (68,69).

Pseudovirus neutralization assay techniques

Similar to PRNT, pseudovirus neutralization assay also requires cell lines that express human ACE2 or animal ACE2, which possesses high similarity to human ACE2 to enable S glycoprotein-ACE2 interaction. Hoffman et al. (54) conducted a pseudovirus neutralization assay in a panel of cell lines and showed that most of the human cells could be used for the assay, including Caco-2 and Calu-3 cells that enable virus cell entry and replication. However, in a replication-incompetent pseudovirus, after receptor binding and cell entry, virus propagation will not occur, so that the neutralization assay by using these types of

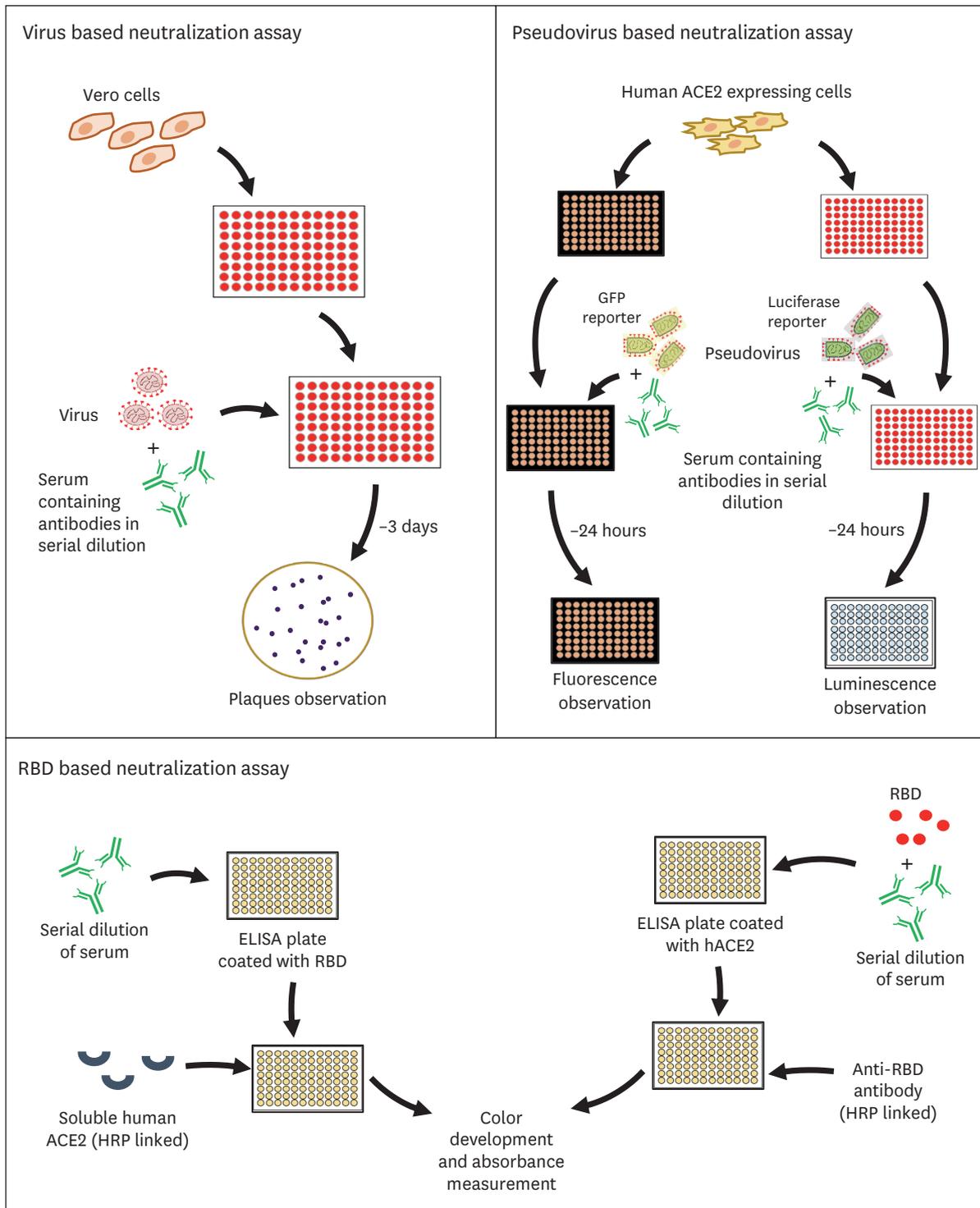


Figure 1. General neutralization assay procedures by using live virus, pseudovirus and recombinant S glycoprotein RBD as epitope sources.

pseudoviruses also can be performed in other cell lines expressing exogenous human ACE2, for instance, HEK293T and BHK-21 cells which are transfected with a plasmid bearing human ACE2 gene to facilitate receptor binding and pseudovirus internalization without the need to support virus replication (64,73) (Fig. 2). Co-expression of exogenous human ACE2 with

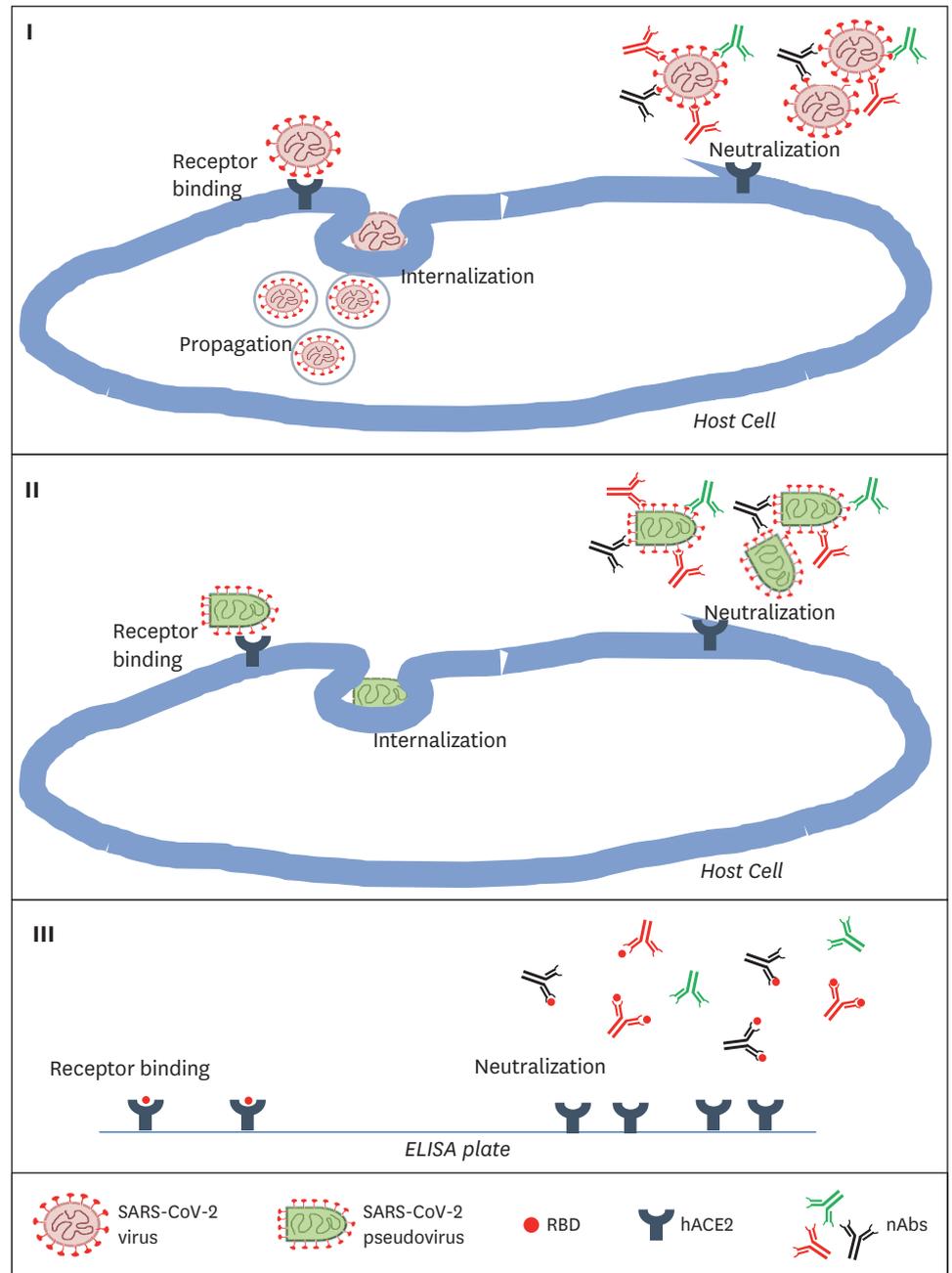


Figure 2. Interaction of hACE 2 with SARS-CoV-2 virus, pseudovirus and RBD either in absence or presence of nAbs I) In the absence of nAbs SARS-CoV-2 virus binds ACE 2 then followed by membrane fusion and cell entry release of its genetic material will lead to virus propagation, II) SARS-CoV-2 pseudovirus is also capable of binding ACE 2 and entering the infected cells non-replicative pseudovirus binds ACE 2 and internalizes, but does not replicate within host cells, III) RBD binds to recombinant human ACE 2 or ACE 2 sub-unit The presence of nAbs blocks ACE 2 interaction with virus, pseudovirus and RBD.

TMPRSS2 was also performed to elevate the degree of pseudovirus internalization (54,74). Pseudovirus-based neutralization assays have been widely conducted along with PRNT (37).

The sensitivity of the pseudovirus neutralization assay will depend on the reporter system applied to analyze cell entry response after receptor binding. Nie et al. (48) synthesized the

SARS-CoV-2 pseudotype by using VSV backbone-bearing luciferase reporter to perform a neutralization assay of serum samples with the serial dilution up to 1:3,200. Luciferase metabolizes its substrate to produce chemiluminescence light that can be analyzed using a luminometer. This reporter offers the greatest sensitivity among other reporters such as fluorescence protein GFP and miRFP. Pseudovirus-based neutralization assays also can be recalled according to the utilization of reporter, for instance, when GFP reporter is used, it can be named as GFP reduction neutralization test (68).

Recombinant S glycoprotein RBD based neutralization assay

RBD plays a crucial role for receptor attachment thereby providing essential epitopes for generation of nAbs. Passariello et al. (80) have successfully generated neutralizing monoclonal Abs by phage display panning using immobilized RBD that show blocking activity by inhibiting RBD-ACE2 interaction and neutralization effect in Vero E6 PRNT. These results emphasize the essential role of RBD to catch nAbs in neutralization assay using RBD peptide. Next, as being considered to be the major epitopes, RBD and its fusion protein also become the main target in vaccine development. A vaccine candidate RBD fused with human IgG Fc (RBD-Fc) has been shown to promote the generation of nAbs against SARS-CoV-2 after immunization in mice. The serum nAbs are capable of inhibiting SARS-CoV-2 pseudovirus cell entry analyzed by luciferase reporter with highest NT50 value obtained from antisera collected at day 35 from mice immunized with 10 µg RBD-Fc. IgG Fc is fused to RBD to increase immunogenicity and half-life by binding to the Fc receptor (81). The presence of both serological anti-SARS-CoV-2 IgG and IgM Abs can be examined by ELISA using recombinant protein derived from SARS-CoV-2 outer proteins including S glycoprotein and its RBD sub-unit, with the application of RBD peptide is expected to represent the measurement of nAbs.

ELISA techniques

The simplest ELISA technique is binding ELISA that utilizes the recombinant RBD. It can be conducted by coating the ELISA plate with this peptide, followed by the addition of antisera and secondary Ab either anti-IgG or anti-IgM. Shen et al. (60) have performed a binding ELISA of prospective donors' serum one day before CP collection by utilizing recombinant S1 RBD at 200 ng/well to detect anti-SARS-CoV-2 Abs. Serial dilution of serum (1:600, 1:1,800, 1:5,400, and 1:16,200) was applied to each well to determine ELISA endpoint dilution Ab titer. All tested CP showed Ab titers equal to or more than 1,800 and were used for study ¹¹. In addition, Li et al. (82) also conducted S-RBD-specific IgG Ab measurement for the quality control of CP. They used 100 ng/well of recombinant RBD polypeptides to detect Abs in a serial diluted CP (1:160, 1:320, 1:640, and 1:1,280). As a result, all CP obtained from 64 donors had a titer higher than 1:160, which fulfills the quality control requirement based on the China national guidelines for COVID-19 CP (82). Moreover, Duan et al. (61) also conducted ELISA to examine the existence of IgG Abs in the donor's plasma before application of CP therapy. However, RBD-based binding ELISA that had been conducted for CP analyses that is associated with nAbs titer (60,61,82) detects total binding Abs (bAbs) and is unable to differentiate between bAbs and nAbs (83-85). Thus, to clarify that RBD-binding Abs also act as nAbs, the ELISA technique can be modified with the utilization of recombinant human ACE2 or soluble ACE2 as decoy receptors to implicate the interaction between RBD and human ACE2. In this ELISA-based neutralization assay, the presence of nAbs in antisera will block RBD-ACE2 interaction. Thus, this ELISA technique is also known as blocking ELISA (34,81). This RBD-ACE2-based blocking ELISA had been applied to measure the neutralization of RBD-human ACE2 by antisera after immunization of mice with

Table 2. Comparison of Ab neutralization assay platforms

Neutralization assay platform	Advantage	Disadvantage
Virus based neutralization assay	Represents the actual virus-nAbs interaction and virus infectivity. Replication competent virus forming visible plaques which correspond to the number of virus particles. Less complicated than pseudotyping.	Virulent SARS-CoV-2 virus needs a BSL-3 laboratory for propagation. Incubation time is longer to observe the visible plaques.
Pseudovirus based neutralization assay	Can be performed in the BSL-2 laboratory. Recombinant virus fused with reporter protein enables high throughput assay with higher sensitivity. Single-shot transfection to generate pseudovirus in each assay eliminates the need of making stock. Can be engineered to be replication incompetent for safety. The conformational structure of pseudovirus surface proteins bears high similarity to that of the native viral proteins.	Pseudovirus conformation depends on the virus backbone. More expensive and complicated than other neutralization assays.
RBD based neutralization assay	Can be performed in a common laboratory. Many commercial kits are available. Enables high throughput assay. Can be conducted without involving cell culture.	Not all nAbs bind to RBD. The least represents the neutralization reaction.

a vaccine candidate RBD-Fc to evaluate its efficacy (81). This blocking ELISA also has been validated to measure nAbs level in COVID-19 patients for serosurveillance that achieves about 99% specificity and about 95% sensitivity (34).

Technically, either RBD or ACE2 can be used for coating the ELISA plate even though ELISA using RBD as coating peptide is easier to be performed to determine nAbs titers. In the procedure using RBD as coating peptide, serum samples in a serial dilution are added to the plate to give the opportunity of RBD-nAbs interaction. The nAbs content in the sample will prevent ACE2 attachment to RBD, leaving recombinant ACE2 to bind free RBD. On the other hand, when recombinant ACE2 is used as a coating peptide, nAbs in the serum samples need to be incubated with recombinant RBD before adding it to the plate. In this case, it is less practical to incubate serial dilution of samples with RBD (68).

ELISA is a widely used technique and is a simpler method in comparison to pseudovirus neutralization assay. Neutralization assay by ELISA offers a lot of advantages because it enables high throughput analysis of serum samples (86). In addition, the protocol does not involve cell culture, allowing the analysis to be performed in ordinary laboratories. Besides, there are many commercial ELISA kits available in the market along with recombinant RBD and ACE2 to be applied in the routine in-house (laboratory) ELISA. Nevertheless, even though the results obtained from this assay also show a correlation with PRNT, there is a possibility that nAbs contained in the samples do not bind RBD as the major epitopes. As the results, it contributes to the lower measured titer. Even though RBD contains important epitopes for generating nAbs, other epitopes may also generate nAbs toward SARS-CoV-2 virus. Chi et al. (87) isolated Abs from CP and identified a neutralizing Ab that, instead of RBD, binds the N-terminal domain of S glycoprotein (82,87). Thus, the assay does not fully represent the actual nAbs titer (Table 2).

WHO establishes anti-SARS-CoV-2 Abs as internal standard and reference panel to create harmonization of nAbs assessments due to various samples and assay types conducted at the different laboratories worldwide (88). This biological standardization will also lead to the determination of the international unit to express the potency of tested Abs or serum samples. To test the internal standard and reference panel candidates, participating laboratories had performed assays based on the protocols developed at their laboratories. Those protocols

involve neutralization assays utilizing live virus, VSV-derived SARS-CoV-2 pseudovirus, HIV lentivirus-derived SARS-CoV-2 pseudovirus, and in-house ELISA. Neutralization assay platforms discussed in this review are summarized in **Figs. 1, 2**, and **Table 2**.

NEUTRALIZATION ASSAYS AGAINST SARS-COV-2 VARIANTS

In line with the emergence of new SARS-CoV-2 variants, the spike S glycoprotein has been reported to be the target of mutation and implicated in the results of neutralization assays. The developed neutralization assays might not detect the current circulating viruses (89,90). Thus, the neutralization assays in each laboratory should be dynamically updated along with the generation of new virus variants. In addition, assay adjustment is needed to investigate the current immunological response of either the infected or vaccinated patients to prevent false-negative results (91,92).

During the COVID-19 pandemic, BSL-3 laboratories play an essential role in surveillance for detecting and isolating the new SARS-CoV-2 virus variants (93). In a BSL-3 laboratory, researchers can isolate and propagate new virus variants to adjust the neutralization test by PRNT. On the other hand, for pseudovirus neutralization assays, adjustment can be carried out by DNA sequence modification of plasmid vectors, such as by inverse PCR, to generate new pseudovirus with modified spike S glycoprotein. For this purpose, complete sequences of new SARS-CoV-2 variants can be downloaded from the GISAID website (26,94-96). Adjustment of current neutralization assays by ELISA will need modification of recombinant RBD or S glycoprotein. The existing plasmid can be engineered according to the GISAID data to produce new mutant proteins for in-house ELISA application. However, commercial ELISA kits for detecting new SARS-CoV-2 variants may not be available yet for a while.

CONCLUSION

The emergence of highly pathogenic viruses demands great efforts to overcome the pandemic by the generation of vaccines, immunological surveillance of patients, as well as the development of effective medications that involve CP. Most of these efforts are associated with the attempts to boost the nAbs level in the human body to encounter the virus attack. It is crucial to determine the nAbs level in plasma to evaluate vaccine efficacy, patient's therapy outcome, and the quality of CP as well. Therefore, serological nAbs neutralization assays become a requirement for researchers and clinicians to develop effective vaccines and medications. As the standard neutralization assay will need the live high pathogenic viruses themselves to be isolated and propagated in either BSL-3 or BSL-4 containments; thus, surrogate neutralization assays that show relevance to the PRNT results are developed as alternatives that offer better safety to be performed at laboratories with lower BSLs.

Nowadays, we are still in the middle of the SARS-CoV-2 pandemic; hence, Ab neutralization assay is in high demand for the assessment of candidate vaccine efficacy, evaluation of CP, and monitoring of the immune response of COVID-19 patients. For these purposes, there are 3 different assay platforms that have been carried out to determine the titer of nAbs based on the epitope sources which are wild-type virus, pseudovirus, and recombinant S glycoprotein RBD-ACE2-based neutralization assays. Each assay possesses its own advantages and disadvantages.

While PRNT using live virus plays as a gold standard assay for the determination of nAbs titers, the limited number of BSL3 laboratories, especially in either developing or under-developed countries, does not enable this assay to be conducted in all regions. The other platforms that correlate to PRNT provide flexibility to perform neutralization assays depending on the available research facilities. Reverse genetic technology enables the generation of modified pseudovirus that provide receptor binding sites to resemble virus-receptor interaction that nAbs can block. Pseudovirus can also be engineered according to the emergence of recent emerging virus mutants along with the additional reporter to facilitate high throughput assay. Furthermore, recombinant DNA technology enables the synthesis of recombinant S glycoprotein RBD and recombinant hACE2 to be applied in a high throughput ELISA that can be done in an ordinary laboratory. However, blocking ELISA is to be considered as surrogate virus neutralization assay, but not binding ELISA.

Overall, based on epitopes sources, PRNT using live SARS-CoV-2 virus is the perfect platform. However, considering the safety level and high throughput application, pseudovirus neutralization assay offers the best platform while maintaining the virus form. Furthermore, blocking ELISA with validated protocol can also be widely applied in the hospitals and smaller healthcare systems since it does not involve cell culture and virus handling. For protocol validation, the reference standard of nAb can be utilized as positive control to clarify the correlation of each assay to PRNT.

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