

## Review

# Detecting SARS-CoV-2 neutralizing immunity: highlighting the potential of split nanoluciferase technology

Sundararaj Stanleyraj Jeremiah, Kei Miyakawa, and Akihide Ryo\*

Department of Microbiology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan

\* Correspondence to: Akihide Ryo, E-mail: [aryo@yokohama-cu.ac.jp](mailto:aryo@yokohama-cu.ac.jp)

Edited by Bing Su

The coronavirus disease 2019 (COVID-19) pandemic has progressed over 2 years since its onset causing significant health concerns all over the world and is currently curtailed by mass vaccination. Immunity acquired against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can be following either infection or vaccination. However, one can never be sure whether the acquired immunity is adequate to protect the individual from subsequent infection because of three important factors: individual variations in humoral response dynamics, waning of protective antibodies over time, and the emergence of immune escape mutants. Therefore, a test that can accurately differentiate the protected from the vulnerable is the need of the hour. The plaque reduction neutralization assay is the conventional gold standard test for estimating the titers of neutralizing antibodies that confer protection. However, it has got several drawbacks, which hinder the practical application of this test for wide-scale usage. Hence, various tests have been developed to detect protective immunity against SARS-CoV-2 that directly or indirectly assess the presence of neutralizing antibodies to SARS-CoV-2 in a lower biosafety setting. In this review, the pros and cons of the currently available assays are elaborated in detail and special focus is put on the scope of the novel split nanoluciferase technology for detecting SARS-CoV-2 neutralizing antibodies.

**Keywords:** COVID-19, neutralizing antibody, immune escape, surrogate virus neutralization test, split nanoluciferase, virus-like particles, immunity passport

### Background

#### *Two years of COVID-19 pandemic*

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) wreaked havoc on a global scale for nearly two years since its outbreak and continues to do so. During the initial months of its onset, with no specific or effective antiviral countermeasures, the disease propagated in successive waves ravaging the naive population. Subsequently, due to the gargantuan efforts of mass vaccination, most people have acquired either post-infection or vaccine-derived immunity against SARS-CoV-2 (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a). By the end of 2021,

>276 million people were confirmed infected and >8.6 billion doses of vaccine have been administered (WHO Coronavirus (COVID-19) Dashboard). While the world is posed to see the light at the end of the tunnel as we brace for milder subsequent waves of infection, many activities such as travel, education, and recreational gatherings, which were either halted or happening virtually, are slowly resuming to the pre-pandemic ways, relying on the natural or artificial immunity acquired against SARS-CoV-2 (Dyer, 2021). In this setting, there are three main factors that influence the protection offered by pre-existing immunity from subsequent reinfection, namely individual variations in humoral response dynamics, waning of protective antibodies over time, and immune escape mutants.

#### *Individual variations in humoral response dynamics*

Subsequent to infection or vaccination, the body mounts both cellular and humoral immune responses against SARS-CoV-2 (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a). Humoral immunity comprises antibodies that only bind to viral epitopes without

Received December 28, 2021. Revised February 22, 2022. Accepted March 1, 2022.

© The Author(s) (2022). Published by Oxford University Press on behalf of *Journal of Molecular Cell Biology*, CEMCS, CAS.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

neutralizing the infectivity of the virion (non-nAbs) and the neutralizing antibodies (nAbs) that are considered protective from reinfection. While a minor subset of individuals do not seroconvert after infection, the majority of those who do (76%–99%) show individual variations in antibody levels (Dan et al., 2021; Wei et al., 2021). Studies have shown that seroconversion following infection is influenced by viral load, disease severity, and age of the individual (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a). Unlike infections, mRNA vaccines have been shown to achieve consistent seroconversion in all recipients (Jackson et al., 2020; Walsh et al., 2020). However, elderly people, people with malignancies or immune disorders, and those on immunosuppressive therapies produce much lower levels of antibodies upon seroconversion following infection or vaccination (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a; Gavriatopoulou et al., 2021; Terpos et al., 2021). Also, mere seroconversion does not reflect protective immunity. Chia et al. (2021) have observed that 12% of the convalescents do not possess significant levels of nAbs despite seroconversion. A similar phenomenon of ‘poor responders’ has also been reported with vaccination. Lake et al. (2022) have reported that 25% of vaccine recipients seroconvert yet do not generate adequate nAbs after receiving two doses of vaccine, but only produce nAbs after receiving the booster dose. Also to be noted is the variation in the protective efficacy offered by different vaccine types (Khoury et al., 2021).

#### *Waning of protective antibodies over time*

Waning of SARS-CoV-2 nAb levels is a well-documented phenomenon and this has been observed with the nAbs acquired by either infection or vaccination. Chia et al. (2021) reported different patterns of nAb waning and documented adequate nAb levels in only 61% of the study subjects 6 months after infection. However, subsequent reports show the presence of nAbs in 98% of the infected individuals after 6 months (Goto et al., 2021). Long-term follow-up studies have shown that despite showing a downward trend, adequate levels of nAbs persist in 89%–97% of individuals 1 year after infection (Haveri et al., 2021; Epaulard et al., 2022; Miyakawa et al., 2022). In the case of vaccines, despite showing a declining trend, nAbs have been reported to persist at least until 6 months after the second dose of mRNA vaccines (Pegu et al., 2021). Despite the claim of >90% protection against infection after 6 months by the mRNA vaccines in clinical trials, real-world studies have shown lesser rates (54%–85%) of protection (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a, b). The protective efficacy of the ChAdOx1 nCoV-19 vaccine has been reported to reduce to somewhere between 43% and 62% after the third month of receiving the second dose (Katikireddi et al., 2022). The presence of detectable levels of vaccine-induced nAbs in serum has not correlated with the claimed protective efficacy of vaccines due to the rise of immune escape mutants.

#### *The problem of immune escape mutants*

The introduction of vaccines to curtail the rampantly spreading virus and the presence of antibodies from previous infection have imposed a selection pressure causing the evolution of immune escape mutants (Krause et al., 2021). These mutants possess one or more mutations in their spike that prevent their neutralization by nAbs generated against the older viral strain by previous infection or vaccines. The immune escape mutants can cause reinfections in the previously infected and breakthrough infection in the vaccinated (Harvey et al., 2021). One of the earliest variants reported to have prominent immune escape was the Beta variant. However, due to increased transmissibility, the Delta variant overtook all others and became the predominant variant in circulation. Studies have shown reduced neutralizing activity by pre-existing nAbs, 55%–69% and 68%–79% to Beta and Delta, respectively, while retaining >90% activity against the original strain (Haveri et al., 2021; Miyakawa et al., 2022). The Mu variant was found to be 1.5–2 times as resistant to neutralization by vaccine and convalescent sera as the Beta variant (Uriu et al., 2021). The more recent Omicron variant is said to possess the highest transmissibility and immune escape. Vaccinated sera were reported to show 40-fold reduction of neutralizing activity against the Omicron variant compared with only 3-fold reduction against the Beta variant (Cele et al., 2022).

#### *Paradigm shift in the purpose of COVID-19 diagnostics*

During the early phase of the pandemic, the priority of diagnostics was given to accurate detection of infection and estimation of seroprevalence. Tests that detect SARS-CoV-2 nucleic acids or antigens and serological tests to detect one or more antibody types were developed for this purpose (Kevadiya et al., 2021). Progression of the pandemic ensued in the reduction of the immune-naïve population and increase in the number of people with acquired immunity. With multiple parameters influencing protective immunity as discussed earlier, one cannot be sure whether an infected or vaccinated individual is indeed protected from the currently circulating variant of SARS-CoV-2. Hence, in the present scenario, more emphasis should be given to assays that can detect protective immunity at both individual and community levels.

#### **Methods to detect protective immunity**

Both cell-mediated immunity and humoral immunity are required to confer protection and the evaluation of the latter is comparatively easier for practical purposes (Guihot et al., 2020). The plaque reduction neutralization test (PRNT) has been the conventional and the gold standard method to evaluate nAbs in sera to viral infections. However, to address the need of the hour in the COVID-19 pandemic, several modifications of the PRNT and other novel methods have been developed to demonstrate nAbs to SARS-CoV-2. These comprise non-cell culture-based assays that indirectly identify the presence of nAbs and cell culture-based assays, which directly demonstrate nAbs with a similar core principle to that of the PRNT (Bewley et al., 2021; Lu et al., 2021). Each of these test platforms has

**Table 1** Characteristics of various assays available to detect nAbs to SARS-CoV-2.

Assay type	Assay	Performance	TAT	Biosafety setting	Throughput	References	
Non-cell culture-based assays	Indirect ELISA	Anti-NP IgG	Low	Rapid	Low	High	<a href="#">Perkmann et al. (2021)</a> ; <a href="#">Sholukh et al. (2021)</a>
		Anti-RBD/SP IgG	Low	Rapid	Low	High	<a href="#">Salazar et al. (2020)</a> ; <a href="#">Peterhoff et al. (2021)</a> ; <a href="#">Rowntree et al. (2021)</a> ; <a href="#">Sholukh et al. (2021)</a> ; <a href="#">Dolscheid-Pommerich et al. (2022)</a>
	sVNT	Low	Rapid	Low	High	<a href="#">Meyer et al. (2020)</a> ; <a href="#">Perera et al. (2021)</a> ; <a href="#">Putcharoen et al. (2021)</a> ; <a href="#">Taylor et al. (2021)</a> ; <a href="#">Valcourt et al. (2021)</a> ; <a href="#">von Rhein et al. (2021)</a>	
Cell culture-based assays	PRNT	High	Slow	High	Low	<a href="#">Lu et al. (2021)</a>	
	FRNT	High	Slow	High	Low	<a href="#">Amanat et al. (2020)</a> ; <a href="#">Case et al. (2020)</a> ; <a href="#">Bewley et al. (2021)</a>	
	rVNT	Intermediate	Intermediate	High	Low	<a href="#">Muruato et al. (2020)</a> ; <a href="#">Xie et al. (2020)</a> ; <a href="#">Ye et al. (2021)</a>	
	pVNT	Intermediate to high	Slow	Regular	High	<a href="#">Kalkeri et al. (2021)</a> ; <a href="#">Sholukh et al. (2021)</a> ; <a href="#">von Rhein et al. (2021)</a>	
	hiVNT	High	Rapid	Regular	High	<a href="#">Miyakawa et al. (2021)</a>	

Assay performance is stratified based on its *r*-values obtained upon comparison with the gold standard PRNT in various studies cited: high ( $r > 0.9$ ), intermediate ( $r = 0.8-0.89$ ), and low ( $r < 0.79$ ). TAT is represented as rapid (<5 h), intermediate (5–24 h), and slow (>24 h). Biosafety requirement is classified as high (requiring BSL-3), regular (requiring BSL-2 in a laminar flow cabinet), and low (can be performed on a tabletop in ambient atmosphere). Throughput is stratified based on the capacity of the assay to run several tests in one set (high) or not (low).

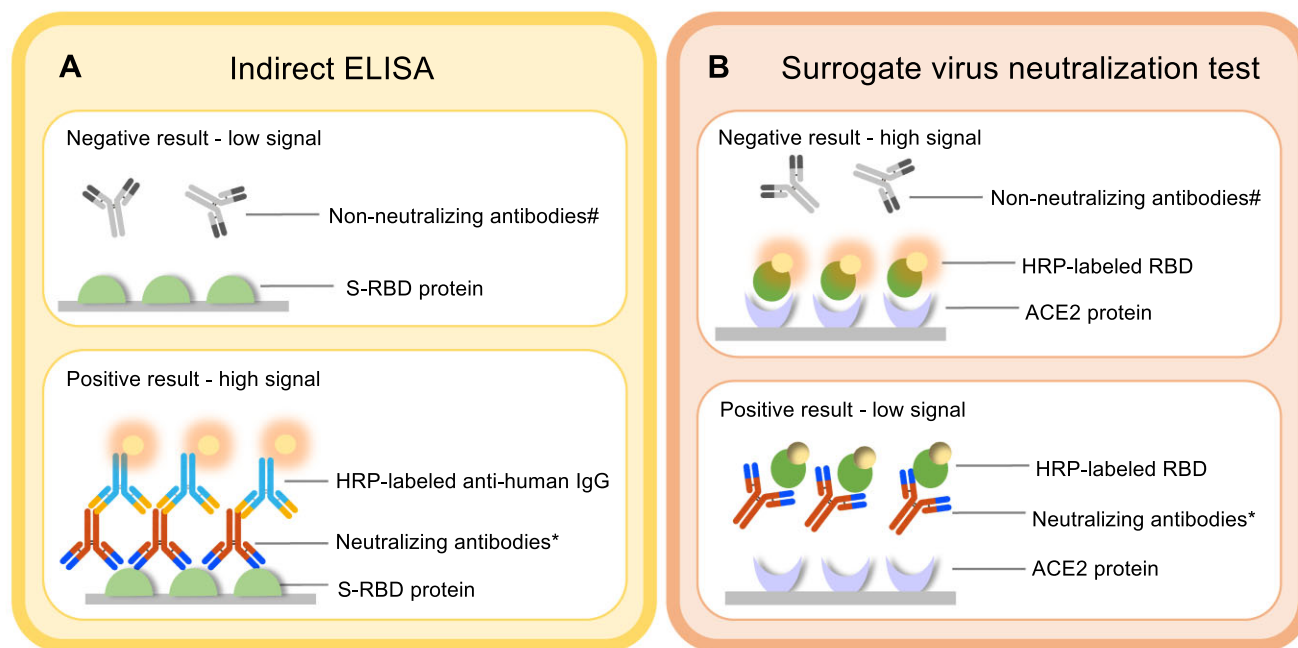
different characteristics about the requirement of biosafety level 3 (BSL-3) facility, ease of use, cost, and turnaround time (TAT). These tests also vary in the reliability of detecting nAbs when compared with the gold standard. An ideal test for use in the field for mass testing of protective immunity should be reliable, rapid, user-friendly, and able to be performed with minimal safety precautions. The different options available for SARS-CoV-2 nAb detection are tabulated in [Table 1](#) and elaborated subsequently.

#### *Non-cell culture-based assays*

*Serological detection of anti-RBD and other IgGs.* In other viral infections, such as hepatitis B, demonstration of adequate titers of antibodies to a specific viral determinant such as the surface antigen is sufficient to denote the presence of neutralizing immunity ([Chaudhari et al., 2008](#)). Since the receptor-binding domain (RBD) was identified as the viral protein crucial for entry, it was initially presumed that antibodies against RBD could directly reflect the neutralizing immunity ([Figure 1A](#)). However, it was subsequently found that all antibodies to the RBD are not necessarily nAbs ([Alsoussi et al., 2020](#)). Also, nAbs that neutralize the virus by binding to epitopes outside the RBD are also known to occur in SARS-CoV-2 infections ([Chi et al., 2020](#)). Hence, the mere demonstration of anti-RBD antibodies could only suggest past/ongoing infection and does not completely represent protective immunity. Various rapid lateral flow assays and state-of-the-art enzyme immunoassays, chemiluminescence immunoassays, and microsphere-based fluorescence

assays are commercially available to detect immunoglobulin G (IgG), other antibody types to RBD, and other specific antigens with high precision ([Kubo et al., 2020](#); [Bray et al., 2021](#)). However, this high diagnostic accuracy of antibody detection does not translate to accurate detection of nAbs ([Patel et al., 2021](#)). [Sholukh et al. \(2021\)](#) evaluated the efficacy of IgG detection ELISA for different antibodies such as anti-nucleocapsid protein (anti-NP), anti-RBD, and anti-spike protein (anti-SP) in reflecting the presence of nAbs and found that anti-NP IgG was the least accurate. [Perkman et al. \(2021\)](#) have also pointed out the inaccuracy of anti-NP IgG's correlation with the neutralizing potential. Some studies show that both anti-RBD IgG and anti-SP IgG fare equally and surprisingly well for this purpose ([Peterhoff et al., 2021](#); [Sholukh et al., 2021](#); [Dolscheid-Pommerich et al., 2022](#)), while other studies report inferior efficacies ([Salazar et al., 2020](#); [Rowntree et al., 2021](#)). A high proportion of false positives can be encountered if anti-RBD IgG and anti-SP IgG are relied on as markers to detect nAbs ([Sholukh et al., 2021](#)).

*sVNT.* During the early months of the pandemic while the biology of the virus was being gradually unraveled, it was found that SARS-CoV-2 interacted through its spike protein with the angiotensin-converting enzyme 2 (ACE2) receptor on target cells to gain entry ([Hoffmann et al., 2020](#)). [Tan et al. \(2020\)](#) speculated that antibodies that bind to the neutralizing epitopes on spike would prevent the interaction of the spike protein with the ACE2 receptor. This was the background for the development of the surrogate virus neutralization test (sVNT) ([Tan et al., 2020](#)).



**Figure 1** Non-cell culture-based assays for detection of SARS-CoV-2 nAbs. **(A)** Indirect ELISA to detect anti-RBD antibodies in sera. Absence of signal denotes the absence of nAbs. **(B)** sVNT. This is a competitive ELISA to specifically detect anti-RBD antibodies that prevent RBD binding to the ACE2 entry receptor. Absence of signal denotes the presence of nAbs. #These are not necessarily non-nAbs. \*These are not necessarily nAbs.

The sVNT is basically a competitive ELISA where the nAbs are allowed to compete with the RBD to bind with ACE2 (Figure 1B). Various sVNT kits have been developed for this purpose, which use either ACE2- (Tan et al., 2020) or RBD-coated wells and the other corresponding antigen conjugate is mixed with the serum and added to the wells. nAbs, if present in the test serum, will compete with RBD to bind with ACE2. This will hamper the antigen conjugate binding to the stationary antigen, leading to reduced signal intensity upon addition of the substrate. On the contrary, the absence of nAbs or the presence of only non-nAbs would allow the antigen conjugate binding, causing an intense signal upon substrate addition.

Among the various sVNT kits developed, the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit developed by GenScript (The Netherlands) is the most widely evaluated and commercially available test. Initial evaluations reported high efficacy of the test with sensitivity >95% and specificity >99% (Tan et al., 2020). Subsequent studies also reported the high accuracy of the cPass™ sVNT for detecting nAbs (Perera et al., 2021; Putharoen et al., 2021; Taylor et al., 2021). However, other studies have reported a lower sensitivity for this assay and stated that the assay fails to detect nAbs when present at lower levels (Meyer et al., 2020; von Rhein et al., 2021).

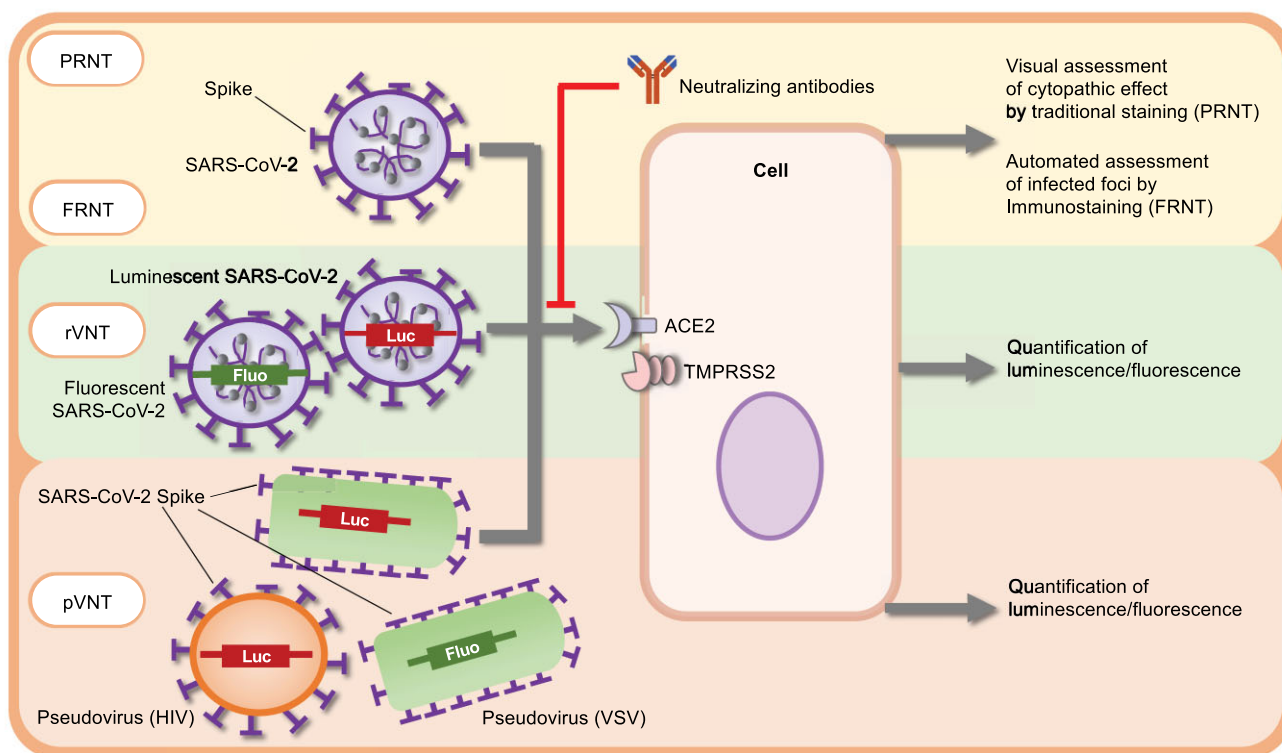
Valcourt et al. (2021) report that cPass™ has high false positive rates >30% due to the erroneous detection of non-nAbs as nAbs. As all anti-RBD antibodies are not necessarily neutralizing (Alsoussi et al., 2020), cPass™, which solely relies on RBD

competition, is prone to causing false positives. False positives could also be encountered in rare instances of autoimmune disorders and vasculopathies, which produce anti-ACE2 autoantibodies (Takahashi et al., 2010). cPass™ can also produce false-negative results. This is because antibodies to other domains of the SP outside the RBD such as the N-terminal domain of S1 (Chi et al., 2020) and the S2 domain (Duan et al., 2005) can also possess neutralizing activity. While cPass™ utilizes the ELISA platform, the 3Flex assay employs the same principle on the fluorescent microsphere-based platform (Angeloni et al., 2021; Cameron et al., 2021).

sVNT offers several advantages. It is simple, user-friendly, does not need a cell culture facility, can be done in a BSL-2 setting, is comparatively inexpensive, and provides results in 3–5 h (Tan et al., 2020). It could also be modified to test dried blood spots with similar TAT (Sancilio et al., 2021). However, it is only an indirect method to hint the presence of nAbs. Considering the fallacies involved in antibody testing, the results of sVNTs should not be directly relied upon (Abbasi 2021). Hence, the scope of the sVNTs could be limited to high-throughput screening of sera eligible for confirmatory PRNT testing (Valcourt et al., 2021).

*Cell culture-based assays with long TAT or the need of BSL-3 facility*

*Authentic live virus neutralization assays.* The PRNT has been used conventionally to detect virus nAbs and is considered the



**Figure 2** Cell culture-based assays for detection of SARS-CoV-2 nAbs. Assays such as PRNT and FRNT use authentic live SARS-CoV-2 to infect target cells and check for cytopathic effect (top panel). rVNT uses recombinant live SARS-CoV-2 incorporated with a genetic element of a reporter molecule such as NanoLuc or Venus fluorescent protein and reads the output using luminescence or fluorescence intensity analysis (middle panel). pVNT uses artificially constructed pseudoviruses using lentiviral or vesicular stomatitis viral elements with exterior SARS-CoV-2 spike and interior NanoLuc reporter gene and reads the output using luminescence (bottom panel). Presence of nAbs diminishes the output signal in either of these test systems.

gold standard assay for this purpose. This test quantifies viral load based on plaques caused by the cytopathic effect of the virus on the cultured cells and provides the nAb levels as 50% neutralization titer (NT<sub>50</sub>) (Figure 2, upper panel). A fixed viral concentration is exposed to sera containing nAbs for a particular time and this mixture is allowed to infect the target cells. After incubation, the virus is disinfected and the cells are fixed with formalin, the cell monolayer is stabilized with agar overlay and stained with a crystal violet dye, and the formed plaques are visualized and counted manually.

The PRNT is highly labor intensive and has a TAT >72–96 h. It has low to medium throughput and is difficult to scale up (Lu et al., 2021). It requires a lot of manual operation, which can result in subjective errors during interpretation. Also, some strains of the virus such as the Delta and Mu do not produce plaques but cause cell-to-cell fusion instead, which is difficult to assess by the PRNT (Mlcochova et al., 2021). These and the other drawbacks of PRNT could be overcome with the focus reduction neutralization test (FRNT), also known as the microneutralization assay (MNA). This assay is a slight variation of the PRNT, especially at the interpretation part. The FRNT/MNA uses immunostaining to visualize infected foci, which can be counted using computer-controlled imagers (Figure 2, upper panel). Au-

tomation of interpretation allows assay miniaturization to increase throughput and reduce the reagent volumes used, manual workload, and subjective errors. However, the FRNT/MNA also has the drawback of requiring a long TAT >48–72 h and requiring BSL-3 setting (Amanat et al., 2020; Case et al., 2020; Bewley et al., 2021).

Apart from the drawbacks faced in technical aspects, the live virus neutralization assays have a major caveat. These tests provide a definite quantitative measurement of nAb activity against SARS-CoV-2, but the correlates of protection of the nAb levels are unknown (Meyer et al., 2020). Through animal infection studies (McMahan et al., 2021) and by backtracking the nAb levels in patients of breakthrough infections (Bergwerk et al., 2021), nAb levels have been shown to have a direct correlation with protection. However, the precise levels of nAbs required to exert definite protection against SARS-CoV-2 infection in the physiological setting are yet to be defined (Kellam and Barclay, 2020). In addition, detection of nAbs that inhibit viral entry alone does not reflect the complete picture of antiviral immunity. Other antibodies that facilitate *in vivo* immune mechanisms, such as opsonization and antibody-dependent cellular cytotoxicity, will be missed if only nAb detection is prioritized (Bournazos and Ravetch, 2017).

*rVNT*. Authentic live virus-based assays depend on traditional staining or immunostaining to read the output. The interpretation could be further enhanced and the TAT could be further reduced by including a reporter gene in the viral genome. Successful viral replication could be assessed and quantified by analyzing the expression of the reporter. Recombinant SARS-CoV-2 engineered to express fluorescent or luminescent reporters enables the performance of live virus neutralization assays that could be interpreted rapidly (Figure 2, middle panel). By substituting the viral ORF7a protein of SARS-CoV-2 with fluorescent (mNeonGreen or Venus) or luminescent (nanoluciferase, NanoLuc) reporter genes, a recombinant reporter virus neutralization test (rVNT) can be developed to accurately detect nAbs (Murua et al., 2020; Ye et al., 2021). Using a recombinant SARS-CoV-2 containing NanoLuc gene at ORF7, Xie et al. (2020) standardized an rVNT assay that could detect nAbs within 5 h. The efficacy of rVNTs has been shown to correlate very well with the PRNT, as they employ real virus and automated reading of the output signals emitted by the reporter simplifies the procedure, reduces the TAT, and enables quantification. They have a high throughput but require a BSL-3 facility.

*pVNT*. Pseudoviruses are constructed over real viruses such as human immunodeficiency virus (HIV) or vesicular stomatitis virus (VSV) by ‘pseudotyping’ the surface of these viruses with the spikes of SARS-CoV-2. These viruses are made replication incompetent by knocking off one or more of their essential genes for replication and replacing with a reporter gene (Schmidt et al., 2020). Replication-competent VSV/SARS-CoV-2 chimeric virus with all aspects like VSV except for the spike of SARS-CoV-2 can also be used for a pseudovirus neutralization test (pVNT) (Case et al., 2020). Pseudoviruses use the same entry mechanism as SARS-CoV-2 and release their contents intracellularly. The reporter gene gets expressed using the target cell transcription machinery and the output could be interpreted by automated instruments. Similar to rVNTs, the presence of nAbs will reduce the output signals (Figure 2, lower panel). Fluorescent reporters such as green fluorescent protein (Case et al., 2020) or chemiluminescent reporters such as firefly luciferase (Nie et al., 2020), renilla luciferase (Oguntuyo et al., 2021), and NanoLuc (Schmidt et al., 2020) are used in pseudoviruses.

In terms of TAT, the VSV-based pVNTs fare better over HIV-based ones as the former replicate quicker than the latter. Despite this, the pVNTs have a TAT of at least 48 h or more. Nanoluciferase-based pVNTs have the problem of high background noise especially in VSV-based systems caused by the leakage of the reporter from cells due to the cytopathic effect by VSV. The main fallacy of pseudoviruses is that they do not ideally represent the biology of the real virus. This is because of the difference in the density of spikes between the two. A greater number of spikes per virion might increase the number of nAbs required to neutralize the virion and vice versa (Schmidt et al., 2020). However, pVNTs have been shown to correlate well with the PRNT (Kalkeri et al., 2021; Sholukh et al., 2021; von Rhein et al., 2021). They can be performed under minimal biosafety

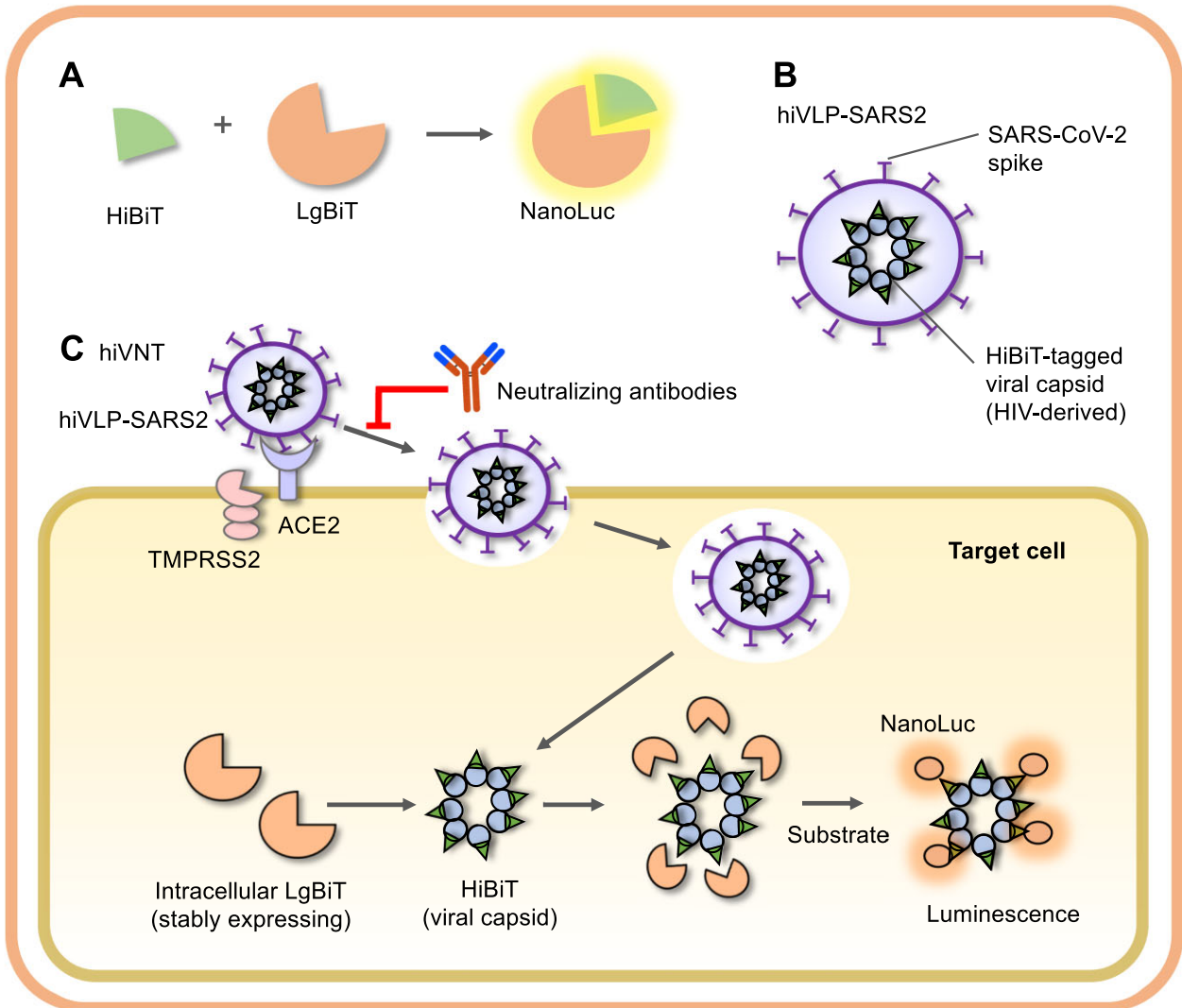
conditions. And the salient advantage of pVNTs is the possibility of switching the spike of the pseudovirus to match that of any new mutant. This feature can help in studying the neutralizing potential of pre-existing nAbs on new mutants of SARS-CoV-2 (Miyakawa et al., 2021).

*Cell culture-based neutralization assays with short TAT and without the need of BSL-3 facility*

*Split NanoLuc technology*. Protein-fragment complementation assay (PCA) utilizes the principle of two or more nonfunctional bits of a reporter protein interacting to produce a full-length functional reporter upon reconstitution (Cabantous et al., 2013; Li et al., 2019). Since their inception in the early 1990s based on reconstituting ubiquitin fragments (Johnsson and Varshavsky, 1994), PCAs rapidly gained popularity as potent tools for studying protein–protein interactions within living cells for the purpose of identifying biochemical networks, determining drug effects, and screening for protein inhibitors. Subsequently, PCAs were developed over various reporter molecules such as enzymes, fluorescent proteins, and bioluminescent proteins (Morell et al., 2009). In recent years, NanoLuc-based assays gained superiority over other reporter assays due to their smaller size, higher stability, lower background activity, and prominent luminescence signal when positive (England et al., 2016). Capitalizing on this technology, a NanoLuc-based PCA was developed by Verhoef et al. (2016), who used NanoLuc split into a 52-amino acid (aa) N-terminal fragment and a 119-aa C-terminal fragment. However, the NanoLuc binary technology (NanoBiT) developed by Dixon et al. (2016) gained commercial success and is popularly known as the HiBiT technology.

The NanoBiT platform utilizes NanoLuc split into two unequal subunits, namely the larger 18-kDa polypeptide called the large bit (LgBiT) and the smaller 1.3-kDa peptide comprising 11-aa called the small bit (SmBiT) (Dixon et al., 2016). However, since the SmBiT showed low affinity ( $K_d > 100 \mu\text{M}$ ) binding to the LgBiT, a novel peptide called the HiBiT with identical proportions to those of the SmBiT was developed to complement the LgBiT. The HiBiT binds to the LgBiT with high affinity ( $K_d = 0.7 \text{ nM}$ ) to produce bright and quantitative luminescence (Schwinn et al., 2018; Figure 3A).

Split NanoLuc technology has gained much significance in studies regarding protein–protein interactions and assessing metabolites involved in cell signaling, molecular biology, and analytical biochemistry (Azad et al., 2014). Owing to its small size, the HiBiT could be tagged to any protein without causing much interference in the interaction of the latter with other proteins (Ranawakage et al., 2019). Hence, the integrated HiBiT reporter faithfully represents target biology. Yet another advantage of the HiBiT reporter is that it could be directly integrated into the genome to express the desired protein. Integration of the HiBiT tag into the endogenous genetic loci of target proteins has been shown to perform better than plasmid overexpression through transfection. And the former also has the advantage of creating edited cell lines that stably express the HiBiT-tagged proteins (Schwinn et al., 2020).



**Figure 3** Novel split NanoLuc system for rapid detection of SARS-CoV-2 nAbs. **(A)** Schematic representation of the principle of the split NanoLuc system. Binding of HiBiT to LgBiT reconstitutes functional NanoLuc capable of emitting luminescence. **(B)** hiVLP-SARS2 are VLPs comprising SARS-CoV-2 spikes on their exterior surface and carry HiBiT-tagged cargo protein within them. **(C)** Schematic representation of the hiVNT assay system. Entry of hiVLP-SARS2 into target cells expressing intracellular LgBiT causes the deposited HiBiT cargo to interact with LgBiT to reconstitute NanoLuc and emit luminescence. Presence of nAbs diminishes the output luminescence.

*Virus interaction assays using split NanoLuc technology.* Having immense potential and pliability to assess protein–protein interaction, the scope of HiBiT technology could be extended to study the interaction of viruses with host cells. The initial steps of viral replication comprise attachment, entry, and release where viruses use their structural proteins to bind to host cell-surface proteins, gaining access across the cell membrane, and releasing their virionic contents into the cytoplasm. Engineered viruses or virus-like particles (VLPs) containing a HiBiT-tagged protein can deliver this cargo into target cells expressing intracellular LgBiT to light up the cells upon successful viral entry. One of the initial studies to use HiBiT technology for viral entry analysis was done by Sasaki et al. (2018), who used VLP-comprising West Nile virus structural proteins tagged with HiBiT

to infect LgBiT-expressing target cells. Successful entry of VLPs into the target cells resulted in the emission of strong luminescence due to the reconstitution of NanoLuc within the infected cells (Sasaki et al., 2018). Subsequently, the split NanoLuc technology was exploited for other viruses using a similar or modified principle (Table 2).

*Tapping the potential of the split NanoLuc system-based assays for detecting SARS-CoV-2 nAbs.* The HiBiT-containing VLP neutralization test (hiVNT) system applies the aforementioned principle for SARS-CoV-2. This system comprises HiBiT-containing VLPs that mimic SARS-CoV-2 (hiVLP-SARS2) and target cells stably expressing LgBiT. The former was generated by cotransfecting two expression vectors: HiBiT-tagged GagPol protein of

**Table 2** Use of the split NanoLuc platform for viral studies.

Assay system	Virus studied	HiBiT-tagged protein	Intended interaction	LgBiT environment	Purpose	Reference
VLPs containing HiBiT cargo	West Nile virus	C protein	Viral entry and release of HiBiT cargo into cells	Target cells expressing intracellular LgBiT	To study flaviviral replication	<a href="#">Sasaki et al. (2018)</a>
	SARS-CoV-2	HIV-1 GagPol	Viral entry and release of HiBiT cargo into cells	Target cells expressing intracellular LgBiT	SARS-CoV-2 entry analysis and to measure nAb levels	<a href="#">Miyakawa et al. (2020)</a>
	SARS-CoV-2	E and M proteins on VLP surface and N protein inside the VLP	Viral entry and release of HiBiT cargo into cells	Target cells expressing intracellular LgBiT	To identify SARS-CoV-2 assembly and entry features	<a href="#">Kumar et al. (2021)</a>
Recombinant viruses containing HiBiT cargo	Infectious bronchitis virus	S or M protein	Viral replication in target cells	Addition to supernatant or cell lysates	To study coronavirus replication and pathogenesis	<a href="#">Liang et al. (2020)</a>
	Bovine leukemia virus	Gag, Env, or Tax	Viral replication in target cells	Addition to supernatant or cell lysates	To identify antiviral drugs	<a href="#">Murakami et al. (2020)</a>
	Oncolytic adenovirus	T2A	Viral replication and persistence in target cells	Target cells expressing intracellular LgBiT; <i>in vivo</i> intraperitoneal injection into infected mice	To study viral biology and viral persistence in infected tumor cells	<a href="#">Gaspar et al. (2020)</a>
	Yellow fever virus	NS1	Viral replication in target cells	Addition to cell lysates	To identify viral entry inhibitor drugs and detect nAbs in immune sera	<a href="#">Sanchez-Velazquez et al. (2020)</a>
	HIV-1	Integrase	Viral replication in target cells	Addition to supernatant	Screening for antiretroviral drugs	<a href="#">Ozono et al. (2020)</a>
Recombinant protein with HiBiT tag	SARS-CoV-2	RBD	Binding with ACE2 on the surface of target cells	Addition to supernatant	To identify drugs that inhibit the viral uptake into cells mediated by binding to ACE2	<a href="#">Lima et al. (2021)</a>
	SARS-CoV-2	RBD	Binding with anti-RBD antibody	Addition to assay well	Serological detection of SARS-CoV-2 antibodies	<a href="#">Rezaei et al. (2021)</a>

HIV-1 and spike protein of SARS-CoV-2 in HEK293 cells were collected from the supernatant 48 h after transfection. Thus, the produced hiVLP-SARS2 had a SARS-CoV-2 spike on their surface and contained only HiBiT-tagged cargo protein within them (Figure 3B). Since these hiVLP-SARS2 are devoid of any genetic element, they can cause a single round of infection and are incapable of replication. The target cells for hiVLP-SARS2 were generated by transfecting LgBiT expression vectors into Vero E6/TMPRSS2 cells. These cells possess both ACE2 and TMPRSS2 on their surface, which are required for SARS-CoV-2 entry. After transfection, the cells that stably expressed LgBiT were selected with hygromycin. These VLPs could enter the target cells using their SARS-CoV-2 spike and deliver the HiBiT into the cytoplasm, where it meets the LgBiT to get reconstituted into functional NanoLuc emitting luminescence that could be quantified (Miyakawa et al., 2020).

The hiVNT assay forms a convenient system to assess the presence of nAbs in sera. The luminescence caused by NanoLuc reconstitution could be prevented by the nAbs that inhibit the VLP entry (Figure 3C). Since the reduction of luminescence intensity is proportional to the amount of nAbs, the assay could be developed to provide quantitative results

(Miyakawa et al., 2020). Since the hiVNT directly demonstrates nAbs with its assay principle identical to that of the PRNT, it is inherently superior to indirect methods of nAb detection such as the sVNT and has a higher degree of correlation to the gold standard PRNT (Miyakawa et al., 2021). The hiVNT also has several advantages over the gold standard PRNT and the pVNT. Since the output signal happens by direct reconstitution of NanoLuc upon hiVLP-SARS2 entry, there is no need for viral replication or transcription for generating the signals. Hence, the TAT for this assay is as short as 3 h, which is the shortest among any of the currently available cell culture-based assays to directly demonstrate nAbs. The VLPs are safe to use in BSL-2 settings. The assay is highly user-friendly as it can be done in a single-step reagent addition process. In addition, the hiVNT assay gives high throughput results as it is performed in 96-well plates and has the scope for further miniaturization into 384- or 1536-well plate format to further increase the throughput (Miyakawa et al., 2021).

Apart from the aforementioned advantages, the hiVNT assay can be reconfigured easily to detect protective immunity to SARS-CoV-2 mutants. The rise and dissemination of mutants is inevitable and with the community spread of every variant,



there is a pressing need to assess whether pre-existing immunity would protect from the new variant. This issue becomes even more crucial if the new variant has the propensity to escape immunity and cause breakthrough infections. In this scenario, the hiVNT can serve as an effective way to analyze vaccine efficacy against new variants. Upon identifying the nucleic acid sequence of the spike of a new variant, hiVLP-SARS2 could be quickly constructed to possess the surface spikes of the new variant. The hiVNT assay was expanded to a panel of seven variants, with the potential to include more variants as they emerge (Miyakawa et al., 2021).

The hiVNT employs NanoLuc, which is a highly sensitive reporter. Hence, the hiVNT assay could detect much lower levels of nAbs than the PRNT. Since the protective threshold of nAb levels is unknown, the biological significance of detecting such low levels of nAbs by hiVNT remains to be found. As the assay platform is licensed by Promega, the cost of reagents and consumables is higher than other assays. Also, the automated detection and quantification of output luminescence requires an expensive analyzer. Establishing and maintaining the target cell lines that stably express intracellular LgBIT is the preliminary requirement for the hiVNT assay. This requires an established cell culture facility and, hence, the assay could not be used as a field test. However, in a centralized testing setting, the hiVNT can be useful in assessing nAbs in test sera for the estimation of individual protection, assessment of herd immunity, and the evaluation of efficacies of vaccines and monoclonal antibodies (mAb cocktails) against established and emerging SARS-CoV-2 variants at a high throughput and short TAT.

### Conclusions and future perspectives

Experiencing 2 years of the COVID-19 pandemic, a majority of the world's population have achieved some form of immunity against SARS-CoV-2. However, due to the interplay of several factors that decide protection, the immune status of a person against the currently circulating variant of SARS-CoV-2 could be confirmed only with a proper test. Although the exact correlates for the threshold of protective nAb levels are currently unknown, the presence of higher levels of nAbs could be taken as positive evidence for protection against severe disease (National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases, 2020a; Bergwerk et al., 2021; McMahan et al., 2021).

A test that accurately detects nAb levels could help in identifying protected individuals who can resume pre-pandemic activities. The test can also demarcate people with inadequate protection to provide them with booster vaccination. It has been shown that booster vaccination helps to increase antibody levels in vaccine nonresponders who generate inadequate protective humoral response (Lake et al., 2022). Also, boosters offer protection against immune escape variants (Garcia-Beltran et al., 2022). For example, elderly people who generate inadequate humoral response or in whom the nAbs

wane rapidly can be identified and targeted for booster administration. On the other hand, younger individuals who have adequate protective immunity need not be prioritized for boosters, thereby rationing the resources to people in need. Since vaccines are known to cause adverse reactions in the younger population, unnecessary administration in the protected individuals can be avoided (Kaneta et al., 2022). Yet, another factor to be considered is the possibility to scale up the assay to perform community-wide testing. If this could be done, the assay can be used in epidemiological analysis for assessing herd immunity in a population. This can help in political decision-making to institute appropriate preventive and control measures.

Various tests are available for detecting neutralizing immunity to SARS-CoV-2. The non-cell culture-based assays such as the sVNT have the advantage of high throughput and ability to scale up to community-wide use in the field setting. However, they have the inherent drawback of reduced accuracy due to the intricacies involved in serological diagnosis. On the other hand, cell culture-based assays have a higher degree of accuracy but require facilities with higher biosafety levels or at least require a clean facility that can handle cell cultures. Taken together along with the TAT factor, the hiVNT appears advantageous over other assays. It can be scaled up for high throughput and easily redesigned to evaluate immunity against a new variant as and when it arises.

Each of the available methods to detect nAbs has its pros and cons and it depends on the health authorities to select the appropriate test that suits the need. As the nAb detection tests vary in their principle, the quantification data provided by the tests can show both inter-assay and batch-to-batch variations. With accumulating data, uniform criteria to obtain a standardized comparison of the different methods must be established. And most importantly, the nAb levels corresponding to the correlates of protection are the need of the hour. Taken together, all these measures can play an important role in reducing the occurrence of reinfections and vaccine-breakthrough infections as we move forward toward the end of the pandemic.

### Acknowledgements

We thank Kenji Yoshihara and Kazuo Horikawa (Yokohama City University, Japan) for their technical assistance and Jesu Priscil Sebastian (Doshisha University, Japan) for his inputs.

### Funding

This work was supported by an Japan Agency for Medical Research and Development (AMED) grant (JP21fk0108104) to A.R.

**Conflict of interest:** none declared.

**Author contributions:** S.S.J. wrote the manuscript and compiled the tables. K.M. wrote the manuscript and drew the figures. A.R. supervised and revised the manuscript. All authors read and approved the final version of the manuscript.

## References

- Abbasi, J. (2021). The flawed science of antibody testing for SARS-CoV-2 immunity. *JAMA* 326, 1781–1782.
- Alsoussi, W.B., Turner, J.S., Case, J.B., et al. (2020). A potentially neutralizing antibody protects mice against SARS-CoV-2 infection. *J. Immunol.* 205, 915–922.
- Amanat, F., White, K.M., Miorin, L., et al. (2020). An in vitro microneutralization assay for SARS-CoV-2 serology and drug screening. *Curr. Protoc. Microbiol.* 58, e108.
- Angeloni, S., Cameron, A., Pecora, N.D., et al. (2021). A rapid, multiplex dual reporter IgG and IgM SARS-CoV-2 neutralization assay for a multiplexed bead-based flow analysis system. *J. Vis. Exp.* e62487.
- Azad, T., Tashakor, A., and Hosseinkhani, S. (2014). Split-luciferase complementary assay: applications, recent developments, and future perspectives. *Anal. Bioanal. Chem.* 406, 5541–5560.
- Bergwerk, M., Gonen, T., Lustig, Y., et al. (2021). Covid-19 breakthrough infections in vaccinated health care workers. *N. Engl. J. Med.* 385, 1474–1484.
- Bewley, K.R., Coombes, N.S., Gagnon, L., et al. (2021). Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and pseudotyped virus neutralization assays. *Nat. Protoc.* 16, 3114–3140.
- Bournazos, S., and Ravetch, J.V. (2017). Diversification of IgG effector functions. *Int. Immunol.* 29, 303–310.
- Bray, R.A., Lee, J.-H., Brescia, P., et al. (2021). Development and validation of a multiplex, bead-based assay to detect antibodies directed against SARS-CoV-2 proteins. *Transplantation* 105, 79–89.
- Cabantous, S., Nguyen, H.B., Pedelacq, J.-D., et al. (2013). A new protein–protein interaction sensor based on tripartite split-GFP association. *Sci. Rep.* 3, 2854.
- Cameron, A., Porterfield, C.A., Byron, L.D., et al. (2021). A multiplex microsphere IgG assay for SARS-CoV-2 using ACE2-mediated inhibition as a surrogate for neutralization. *J. Clin. Microbiol.* 59, e02489-20.
- Case, J.B., Rothlauf, P.W., Chen, R.E., et al. (2020). Neutralizing antibody and soluble ACE2 inhibition of a replication-competent VSV-SARS-CoV-2 and a clinical isolate of SARS-CoV-2. *Cell Host Microbe* 28, 475–485.e5.
- Cele, S., Jackson, L., Khoury, D.S., et al. (2022). Omicron extensively but incompletely escapes Pfizer BNT162b2 neutralization. *Nature* 602, 654–656.
- Chaudhari, C.N., Bhagat, M.R., Shah, T., et al. (2008). Antibody to hepatitis B surface antigen in vaccinated health care workers. *Med. J. Armed Forces India* 64, 329–332.
- Chi, X., Yan, R., Zhang, J., et al. (2020). A neutralizing human antibody binds to the N-terminal domain of the spike protein of SARS-CoV-2. *Science* 369, 650–655.
- Chia, W.N., Zhu, F., Ong, S.W.X., et al. (2021). Dynamics of SARS-CoV-2 neutralising antibody responses and duration of immunity: a longitudinal study. *Lancet Microbe* 2, e240–e249.
- Dan, J.M., Mateus, J., Kato, Y., et al. (2021). Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. *Science* 371, eabf4063.
- Dixon, A.S., Schwinn, M.K., Hall, M.P., et al. (2016). NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* 11, 400–408.
- Dolscheid-Pommerich, R., Bartok, E., Renn, M., et al. (2022). Correlation between a quantitative anti-SARS-CoV-2 IgG ELISA and neutralization activity. *J. Med. Virol.* 94, 388–392.
- Duan, J., Yan, X., Guo, X., et al. (2005). A human SARS-CoV neutralizing antibody against epitope on S2 protein. *Biochem. Biophys. Res. Commun.* 333, 186–193.
- Dyer, O. (2021). Covid-19: two thirds in India carry antibodies, while research suggests country's death toll is 10 times official figure. *BMJ* 374, n1856.
- England, C.G., Ehlerding, E.B., and Cai, W. (2016). NanoLuc: a small luciferase is brightening up the field of bioluminescence. *Bioconjugate Chem.* 27, 1175–1187.
- Epaulard, O., Buisson, M., Nemoz, B., et al. (2022). Persistence at one year of neutralizing antibodies after SARS-CoV-2 infection: influence of initial severity and steroid use. *J. Infect.* 84, 418–467.
- Garcia-Beltran, W.F., St Denis, K.J., Hoelzemer, A., et al. (2022). mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* 185, 457–466.e4.
- Gaspar, N., Zambito, G., Dautzenberg, I.J.C., et al. (2020). NanoBiT system and hydrofurimazine for optimized detection of viral infection in mice—a novel in vivo imaging platform. *Int. J. Mol. Sci.* 21, 5863.
- Gavriatopoulou, M., Terpos, E., Ntanasis-Stathopoulos, I., et al. (2021). Poor neutralizing antibody responses in 106 patients with WM after vaccination against SARS-CoV-2: a prospective study. *Blood Adv.* 5, 4398–4405.
- Goto, A., Go, H., Miyakawa, K., et al. (2021). Sustained neutralizing antibodies 6 months following infection in 376 Japanese COVID-19 survivors. *Front. Microbiol.* 12, 661187.
- Guihot, A., Litvinova, E., Autran, B., et al. (2020). Cell-mediated immune responses to COVID-19 infection. *Front. Immunol.* 11, 1662.
- Harvey, W.T., Carabelli, A.M., Jackson, B., et al. (2021). SARS-CoV-2 variants, spike mutations and immune escape. *Nat. Rev. Microbiol.* 19, 409–424.
- Haveri, A., Ekström, N., Solastie, A., et al. (2021). Persistence of neutralizing antibodies a year after SARS-CoV-2 infection in humans. *Eur. J. Immunol.* 51, 3202–3213.
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280.e8.
- Jackson, L.A., Anderson, E.J., Roupael, N.G., et al. (2020). An mRNA vaccine against SARS-CoV-2—preliminary report. *N. Engl. J. Med.* 383, 1920–1931.
- Johnsson, N., and Varshavsky, A. (1994). Split ubiquitin as a sensor of protein interactions in vivo. *Proc. Natl Acad. Sci.* 91, 10340–10344.
- Kalker, R., Cai, Z., Lin, S., et al. (2021). SARS-CoV-2 spike pseudoviruses: a useful tool to study virus entry and address emerging neutralization escape phenotypes. *Microorganisms* 9, 1744.
- Kaneta, K., Yokoi, K., Jojima, K., et al. (2022). Young male with myocarditis following mRNA-1273 vaccination against coronavirus disease-2019 (COVID-19). *Circ. J.* 86, 721.
- Katikireddi, S.V., Cerqueira-Silva, T., Vasileiou, E., et al. (2022). Two-dose ChAdOx1 nCoV-19 vaccine protection against COVID-19 hospital admissions and deaths over time: a retrospective, population-based cohort study in Scotland and Brazil. *Lancet North Am. Ed.* 399, 25–35.
- Kellam, P., and Barclay, W. (2020). The dynamics of humoral immune responses following SARS-CoV-2 infection and the potential for reinfection. *J. Gen. Virol.* 101, 791–797.
- Kevadiya, B.D., Machhi, J., Herskovitz, J., et al. (2021). Diagnostics for SARS-CoV-2 infections. *Nat. Mater.* 20, 593–605.
- Khoury, D.S., Cromer, D., Reynaldi, A., et al. (2021). Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat. Med.* 27, 1205–1211.
- Krause, P.R., Fleming, T.R., Longini, I.M., et al. (2021). SARS-CoV-2 variants and vaccines. *N. Engl. J. Med.* 385, 179–186.
- Kubo, S., Ohtake, N., Miyakawa, K., et al. (2020). Development of an automated chemiluminescence assay system for quantitative measurement of multiple anti-SARS-CoV-2 antibodies. *Front. Microbiol.* 11, 628281.
- Kumar, B., Hawkins, G.M., Kicmal, T., et al. (2021). Assembly and entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2): evaluation using virus-like particles. *Cells* 10, 853.
- Lake, D.F., Roeder, A.J., Gonzalez-Moa, M.J., et al. (2022). Third COVID-19 vaccine dose boosts neutralising antibodies in poor responders. *Commun. Med.* 2, 85.
- Li, P., Wang, L., and Di, L.-J. (2019). Applications of protein fragment complementation assays for analyzing biomolecular interactions and biochemical networks in living cells. *J. Proteome Res.* 18, 2987–2998.
- Liang, X.Y., Zhu, Q.C., Liang, J.Q., et al. (2020). Development of HiBiT-tagged recombinant infectious bronchitis coronavirus for efficient in vitro and in vivo viral quantification. *Front. Microbiol.* 11, 2100.

- Lima, M.A., Skidmore, M., Khanim, F., et al. (2021). Development of a nanoluciferase based assay to measure the binding of SARS-CoV-2 spike receptor binding domain to ACE-2. *Biochem. Biophys. Res. Commun.* 534, 485–490.
- Lu, Y., Wang, J., Li, Q., et al. (2021). Advances in neutralization assays for SARS-CoV-2. *Scand. J. Immunol.* 94, e13088.
- McMahan, K., Yu, J., Mercado, N.B., et al. (2021). Correlates of protection against SARS-CoV-2 in rhesus macaques. *Nature* 590, 630–634.
- Meyer, B., Reimerink, J., Torriani, G., et al. (2020). Validation and clinical evaluation of a SARS-CoV-2 surrogate virus neutralisation test (sVNT). *Emerg. Microbes Infect.* 9, 2394–2403.
- Miyakawa, K., Jeremiah, S.S., Kato, H., et al. (2021). Rapid detection of neutralizing antibodies to SARS-CoV-2 variants in post-vaccination sera. *J. Mol. Cell Biol.* 13, 918–920.
- Miyakawa, K., Jeremiah, S.S., Ohtake, N., et al. (2020). Rapid quantitative screening assay for SARS-CoV-2 neutralizing antibodies using HiBiT-tagged virus-like particles. *J. Mol. Cell Biol.* 12, 987–990.
- Miyakawa, K., Kubo, S., Jeremiah, S.S., et al. (2022). Persistence of robust humoral immune response in coronavirus disease 2019 convalescent individuals over 12 months after infection. *Open Forum Infect. Dis.* 9, ofab626.
- Mlcochova, P., Kemp, S., Dhar, M.S., et al. (2021). SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. *Nature* 599, 114–119.
- Morell, M., Ventura, S., and Avilés, F.X. (2009). Protein complementation assays: approaches for the in vivo analysis of protein interactions. *FEBS Lett.* 583, 1684–1691.
- Murakami, H., Yajima, Y., Sato, F., et al. (2020). Development of multipurpose recombinant reporter bovine leukemia virus. *Virology* 548, 226–235.
- Muruato, A.E., Fontes-Garfias, C.R., Ren, P., et al. (2020). A high-throughput neutralizing antibody assay for COVID-19 diagnosis and vaccine evaluation. *Nat. Commun.* 11, 4059.
- National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases. (2020a). Science brief: SARS-CoV-2 infection-induced and vaccine-induced immunity. In: CDC COVID-19 Science Briefs. Atlanta (GA): Centers for Disease Control and Prevention (USA).
- National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases. (2020b). Science brief: COVID-19 vaccines and vaccination. In: CDC COVID-19 Science Briefs. Atlanta (GA): Centers for Disease Control and Prevention (USA).
- Nie, J., Li, Q., Wu, J., et al. (2020). Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. *Nat. Protoc.* 15, 3699–3715.
- Oguntuyo, K.Y., Stevens, C.S., Hung, C.T., et al. (2021). Quantifying absolute neutralization titers against SARS-CoV-2 by a standardized virus neutralization assay allows for cross-cohort comparisons of COVID-19 sera. *mBio* 12, e02492-20.
- Ozono, S., Zhang, Y., Tobiome, M., et al. (2020). Super-rapid quantitation of the production of HIV-1 harboring a luminescent peptide tag. *J. Biol. Chem.* 295, 13023–13030.
- Patel, E.U., Bloch, E.M., Clarke, W., et al. (2021). Comparative performance of five commercially available serologic assays to detect antibodies to SARS-CoV-2 and identify individuals with high neutralizing titers. *J. Clin. Microbiol.* 59, e02257-20.
- Pegu, A., O'Connell, S., Schmidt, S.D., et al. (2021). Durability of mRNA-1273 vaccine-induced antibodies against SARS-CoV-2 variants. *Science* 373, 1372–1377.
- Perera, R., Ko, R., Tsang, O.T.Y., et al. (2021). Evaluation of a SARS-CoV-2 surrogate virus neutralization test for detection of antibody in human, canine, cat, and hamster sera. *J. Clin. Microbiol.* 59, e02504-20.
- Perkmann, T., Koller, T., Perkmann-Nagele, N., et al. (2021). Spike protein antibodies mediate the apparent correlation between SARS-CoV-2 nucleocapsid antibodies and neutralization test results. *Microbiol. Spectr.* 9, e0021821.
- Peterhoff, D., Glück, V., Vogel, M., et al. (2021). A highly specific and sensitive serological assay detects SARS-CoV-2 antibody levels in COVID-19 patients that correlate with neutralization. *Infection* 49, 75–82.
- Putcharoen, O., Wacharapluesadee, S., Chia, W.N., et al. (2021). Early detection of neutralizing antibodies against SARS-CoV-2 in COVID-19 patients in Thailand. *PLoS One* 16, e0246864.
- Ranawakage, D.C., Takada, T., and Kamachi, Y. (2019). HiBiT-qIP, HiBiT-based quantitative immunoprecipitation, facilitates the determination of antibody affinity under immunoprecipitation conditions. *Sci. Rep.* 9, 6895.
- Rezaei, R., Surendran, A., Singaravelu, R., et al. (2021). Detection of SARS-CoV-2 receptor-binding domain antibody using a HiBiT-based bioreporter. *J. Vis. Exp.* e62488.
- Rowntree, L.C., Chua, B.Y., Nicholson, S., et al. (2021). Robust correlations across six SARS-CoV-2 serology assays detecting distinct antibody features. *Clin. Immunol. Immunopathol.* 10, e1258.
- Salazar, E., Kuchipudi, S.V., Christensen, P.A., et al. (2020). Convalescent plasma anti-SARS-CoV-2 spike protein ectodomain and receptor-binding domain IgG correlate with virus neutralization. *J. Clin. Invest.* 130, 6728–6738.
- Sanchez-Velazquez, R., de Lorenzo, G., Tandavanitj, R., et al. (2020). Generation of a reporter yellow fever virus for high throughput antiviral assays. *Antiviral Res.* 183, 104939.
- Sancilio, A.E., D'Aquila, R.T., McNally, E.M., et al. (2021). A surrogate virus neutralization test to quantify antibody-mediated inhibition of SARS-CoV-2 in finger stick dried blood spot samples. *Sci. Rep.* 11, 15321.
- Sasaki, M., Anindita, P.D., Phongphaew, W., et al. (2018). Development of a rapid and quantitative method for the analysis of viral entry and release using a NanoLuc luciferase complementation assay. *Virus Res.* 243, 69–74.
- Schmidt, F., Weisblum, Y., Muecksch, F., et al. (2020). Measuring SARS-CoV-2 neutralizing antibody activity using pseudotyped and chimeric viruses. *J. Exp. Med.* 217, e20201181.
- Schwinn, M.K., Machleidt, T., Zimmerman, K., et al. (2018). CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* 13, 467–474.
- Schwinn, M.K., Steffen, L.S., Zimmerman, K., et al. (2020). A simple and scalable strategy for analysis of endogenous protein dynamics. *Sci. Rep.* 10, 8953.
- Sholukh, A.M., Fiore-Gartland, A., Ford, E.S., et al. (2021). Evaluation of cell-based and surrogate SARS-CoV-2 neutralization assays. *J. Clin. Microbiol.* 59, e0052721.
- Takahashi, Y., Haga, S., Ishizaka, Y., et al. (2010). Autoantibodies to angiotensin-converting enzyme 2 in patients with connective tissue diseases. *Arthritis Res. Ther.* 12, R85.
- Tan, C.W., Chia, W.N., Qin, X., et al. (2020). A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2–spike protein–protein interaction. *Nat. Biotechnol.* 38, 1073–1078.
- Taylor, S.C., Hurst, B., Charlton, C.L., et al. (2021). A new SARS-CoV-2 dual-purpose serology test: highly accurate infection tracing and neutralizing antibody response detection. *J. Clin. Microbiol.* 59, e02438-20.
- Terpos, E., Gavriatopoulou, M., Fotiou, D., et al. (2021). Poor neutralizing antibody responses in 132 patients with CLL, NHL and HL after vaccination against SARS-CoV-2: a prospective study. *Cancers* 13, 4480.
- Uriu, K., Kimura, I., Shirakawa, K., et al. (2021). Neutralization of the SARS-CoV-2 Mu variant by convalescent and vaccine serum. *N. Engl. J. Med.* 385, 2397–2399.
- Valcourt, E.J., Manguiat, K., Robinson, A., et al. (2021). Evaluation of a commercially-available surrogate virus neutralization test for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). *Diagn. Microbiol. Infect. Dis.* 99, 115294.
- Verhoef, L., Mattioli, M., Ricci, F., et al. (2016). Multiplex detection of protein–protein interactions using a next generation luciferase reporter. *Biochim. Biophys. Acta* 1863, 284–292.

- von Rhein, C., Scholz, T., Henss, L., et al. (2021). Comparison of potency assays to assess SARS-CoV-2 neutralizing antibody capacity in COVID-19 convalescent plasma. *J. Virol. Methods* 288, 114031.
- Walsh, E.E., Frenck, R.W., Falsey, A.R., et al. (2020). Safety and immunogenicity of two RNA-based Covid-19 vaccine candidates. *N. Engl. J. Med.* 383, 2439–2450.
- Wei, J., Matthews, P.C., Stoesser, N., et al. (2021). Anti-spike antibody response to natural SARS-CoV-2 infection in the general population. *Nat. Commun.* 12, 6250.
- WHO Coronavirus (COVID-19) Dashboard. <https://covid19.who.int> (date last accessed December 9, 2021).
- Xie, X., Muruato, A.E., Zhang, X., et al. (2020). A nanoluciferase SARS-CoV-2 for rapid neutralization testing and screening of anti-infective drugs for COVID-19. *Nat. Commun.* 11, 5214.
- Ye, C., Chiem, K., Park, J.-G., et al. (2021). Analysis of SARS-CoV-2 infection dynamic *in vivo* using reporter-expressing viruses. *Proc. Natl Acad. Sci.* 118, e2111593118.