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Application Note

Rapid quantitative screening assay for SARS-CoV-2 neutralizing antibodies using HiBiT-tagged virus-like particles

Due to the unavailability of any specific countermeasure, the constantly spreading COVID-19 pandemic could only be partially and temporarily slowed down by implementing regional lockdowns that force people to stay at home and prevent their movement. With the progression of the pandemic, a considerable subset of the population would have acquired post-infection immunity and the tests that reveal the post-infection immune status of individuals are the need of the hour. A credible test, which accurately identifies the protected, can offer an immunity passport for the individual to be freed from the lockdown and resume routine activities without the fear of getting infected. At present, the semi-quantitative neutralization test (NT) and the quantitative plaque reduction neutralization test (PRNT) identifying the presence of anti-SARS-CoV-2 neutralizing antibodies (nAbs) are the only foolproof methods available for this purpose. However, practical feasibility of these highly specific tests is weighed down by drawbacks such as low throughput, long turnaround time (TAT), and the need for a specialized laboratory setup with biosafety level 3 (BSL3) facilities to handle the live viruses used in these tests (Cao et al., 2020).

To overcome these hurdles, pseudovirus-based NT (Nie et al., 2020), surrogate serodiagnostic tests (sVNT) (Tan et al., 2020), and single-cell RNA sequencing (Cao et al., 2020) are being studied. However, a simple, convenient, rapid, and high-throughput test capable of directly detecting nAbs with high specificity, which could act as an ideal alternative to the neutralization assay, is yet to be developed (Ozcurumez et al., 2020). Virus-like particles (VLPs) are self-assembling, non-replicating, non-pathogenic entities of similar size and conformation as that of infectious virions. VLPs can be generated to possess the surface proteins of any kind of viruses on their membrane devoid of the viral nucleic acids. This genome-free feature of VLPs overrides the need for BSL3 facilities while handling but has the drawback of difficulty in quantifying its cell entry and membrane fusion.

HiBiT is an 11-amino acid peptide tag that can be attached to any protein-of-interest. LgBiT, the counterpart of NanoLuc luciferase is complementary to HiBiT and binds to the latter to produce a highly active luciferase enzyme. HiBiT-tagged proteins can be easily detected and quantified based on bioluminescence using the Nano-Glo assay system. HiBiT technology offers the advantages of high sensitivity, convenience of a single-reagent-addition step, and short TAT of only a few minutes. In this report, we have developed a HiBiT-VLP-based neutralization test (hiVNT) that can readily detect SARS-CoV-2 nAbs (Figure 1A).

To establish hiVNT, we inserted the HiBiT tag to the HIV-1 Gag-Pol protein, a minimal subunit to produce VLPs. After testing several prototypes, we found that HiBiT tag insertion at the C-terminal region of the capsid gene in Gag-Pol performed best (Supplementary Figure S1A). HiBiT tag can bind with high affinity to its complementary larger subunit LgBiT and form luciferase (NanoLuc) (Sasaki et al., 2018). As expected, HiBiT-containing VLPs (hereafter designated as hiVLP-SARS2) could emit light upon addition of recombinant LgBiT and Furimazine substrate. As the capsid antigen carried the HiBiT tag, the luminescence intensity correlated with the amount of capsid antigen (Supplementary Figure S1B). By co-transfecting Gag-Pol-HiBiT and SARS-CoV-2 spike expression vectors, we generated hiVLP-SARS2 and confirmed their expression in cell and virus lysates (Supplementary Figure S1C).

Since SARS-CoV-2 can infect VeroE6/TMPRSS2 cells with high efficiency (Matsuyama et al., 2020), we next generated LgBiT-expressing VeroE6/TMPRSS2 cells. We noticed a robust increase in NanoLuc activity when the LgBiT-expressing VeroE6/TMPRSS2 cells were treated with hiVLP-SARS2 for 3 h (Figure 1B; Supplementary Figure S1D). We further demonstrated the drop in luminescence signals upon pretreatment with a TMPRSS2 inhibitor (Camostat mesylate) and also with anti-Spike neutralizing monoclonal antibody (Supplementary Figure S1E). We did not observe any reduction in the luminescence signals of hiVLP carrying VSVg envelope (Supplementary Figure S1E), suggesting that hiVLP-SARS2 enters the

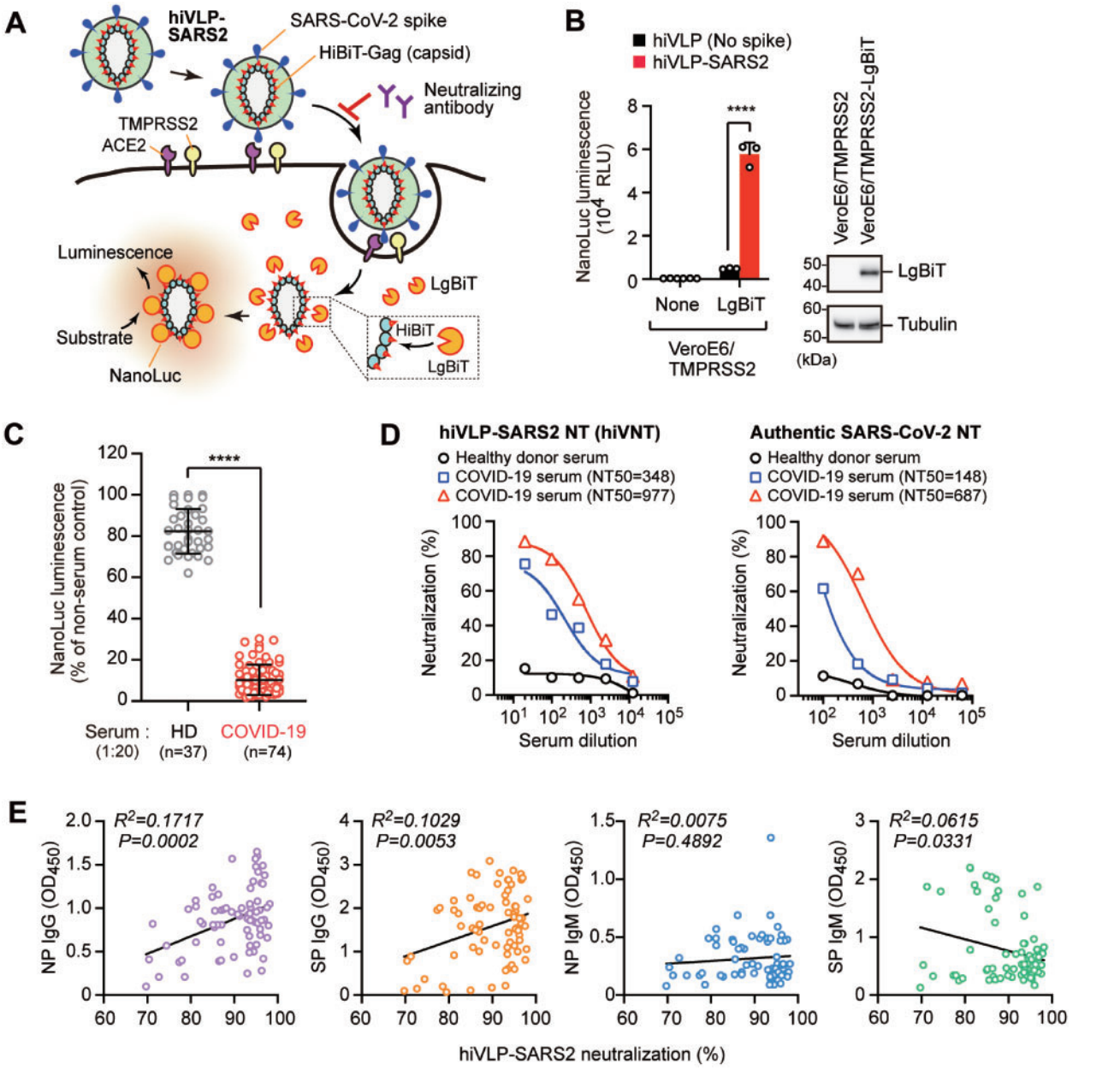

Figure 1 Rapid quantitative assay for SARS-CoV-2 nAbs. (**A**) Schematic representation of hiVLP-SARS2-based neutralization test (hiVNT). (**B**) Detection of cell entry of hiVLP-SARS2. VeroE6/TMPRSS2-LgBiT cells were inoculated with hiVLP-SARS2 for 3 h, after which the intracellular NanoLuc luminescence was measured. The expression of HaloTag-fused LgBiT in target cells was confirmed by immunoblotting analysis. $^{****}P < 0.0001$. (**C**) NanoLuc luminescence of cells inoculated with hiVLP-SARS2 in either healthy donor (HD, $n = 37$) or convalescent COVID-19 patient sera ($n = 74$) for 3 h. $^{****}P < 0.0001$. (**D**) hiVNT is consistent with the NT using authentic SARS-CoV-2. Data of healthy donor and two representative patients are shown with NT titer (NT50). (**E**) Correlation between serological test and neutralization activity of convalescent COVID-19 patient serum. $n = 74$ samples. NP, nucleocapsid protein; SP, spike protein.

target cells through the interactions between viral spike and cellular receptors in a similar way as that of authentic SARS-CoV-2.

We next tested whether our newly developed hiVLP-SARS2 system could detect nAbs in the serum of COVID-19 patients. The neutralization assay was carried out in accordance with the following steps. VeroE6/TMPRSS2-LgBiT cells were seeded on 96-well white polystyrene plates at a density of 10^4 cells/well 1 day prior to inoculation. Decomplemented sera derived from convalescent COVID-19 patients were mixed with hiVLP-SARS2 and the mixture was inoculated to VeroE6/TMPRSS2-LgBiT cells. Three hours later, the cells were washed with PBS and measured for their NanoLuc luminescence. We found that all the tested patients' sera could

block the entry of hiVLP-SARS2 in varying degrees (Figure 1C), suggesting the presence of nAbs against SARS-CoV-2. Two convalescent sera and one healthy donor serum were tested by hiVNT and authentic SARS-CoV-2 NT only to show that all the samples gave similar results in both tests (Figure 1D), suggesting that hiVNT performance is in concordance with the NT using authentic SARS-CoV-2. Since surrogate antibody detection is being widely studied as an alternative to NT, we wanted to check the correlation of nAbs detected by hiVNT with antibody titers detected by ELISA. We matched the results of hiVNT against the antibody titers detected by ELISA in 74 COVID-19-positive sera. nAb levels were found to correlate with IgG antibodies, but not IgM antibodies, against both the nucleocapsid and spike proteins (Figure 1E). Taken together, our novel hiVNT could be useful for rapid detection of nAbs in the sera of individuals recovered from COVID-19.

The hiVNT assay principle is similar to conventional neutralization assays and is based on viral entry and membrane fusion with measurement of NanoLuc luciferase activity to simplify the outcome. Considering the drawbacks of NT and PRNT, tests that may confer immunity passport to individuals are the need of the hour and different platforms are being exploited for this purpose, even though more detailed serological studies are needed. Pseudovirus-based NTs are the popular alternatives to detect nAbs, as they overcome the need for BSL3 facility and have a high-throughput scale (Muruato et al., 2020). These tests employ pseudoviruses, which possess an envelope with SARS-CoV-2 spike proteins and also incorporate a reporter gene in their genome. The test depends on the expression of the reporter gene in the target cells infected with pseudovirus, which takes 24–48 h to be detected. In comparison, our hiVNT does not involve gene expression and thus shortens the TAT to only ∼3 h. Single-cell sequencing might have similar advantages (Cao et al., 2020) but the need for specialized and expensive equipment hinders its practical application.

Measurement of antiviral antibodies in the serum of convalescent COVID-19 patients is being studied for its potential to act as a surrogate marker to reflect the presence of SARS-CoV-2 nAbs (Tan et al., 2020). Our findings in a small sample size ($n = 74$) reveal that IgG could serve as a better surrogate marker than IgM. However, the use of serodiagnostics for nAb detection could have a few inherent drawbacks. Although all COVID-19 infections elicit a humoral immune response, the presence of antibodies does not reflect immunity. Also, mild COVID-19 infections elicit very low humoral response, which might not be detected by serological tests (Ozcurumez et al., 2020). In this setting, surrogate antibody detection tests can produce erroneous results. Moreover, SARS-CoV-2 infects host cells through receptor binding domain (RBD) within S1 subunit in spike protein on the surface of viral particles that bind to host surface angiotensin-converting enzyme-2 receptor. ELISA kits to detect anti-RBD antibodies use *in vitro* generated spike protein and its derivatives to detect nAbs in serum. However, since spike proteins undergo glycosylation and oligomerization *in vivo* (Xiao et al., 2004; Walls et al., 2020), the performance of *in vitro* generated spike proteins may vary depending on the manufacturing method, as well as their clinical significance. Also, nAbs that target other regions of spike protein to suppress the function of fusion peptide of S2 subunit may exist, which could go undetected in S1 and RBD-based ELISA detection (Chi et al., 2020).

In this study, we established the hiVNT, a simple, high-throughput assay system for the quantitative and rapid determination of SARS-CoV-2 nAbs in the sera of individuals after recovery from symptomatic or subclinical COVID-19. The hiVNT system allows for BSL2-compliant testing and gives quantitative results at ultra-high-throughput. The luciferase-fragment complementation assay has been demonstrated to exhibit superior sensitivity with scope for further miniaturization into 384- or 1536-well plate format. Based on our findings, we believe that the hiVNT, by utilizing the NanoLuc technology, can be instrumental in identifying individuals with protective immunity, carrying out epidemiological studies on population susceptibility, modeling virus propagation, and assessing convalescent plasma for therapy and vaccine evaluation. Encouragingly, all these can be achieved on a high-throughput platform, with short TAT, and in a lower biosafety setting.

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