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Identification of SARS-CoV-2 Neutralizing Antibody with Pseudotyped Virus-based Test on HEK-293T hACE2 Cells

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Abstract

Neutralizing antibodies (NAbs) are of particular importance because they can prevent binding of the receptor binding domain (RBD) of the spike protein (S protein) to the angiotensin-converting enzyme 2 (ACE2) receptor present at the surface of human cells, preventing virus entry into the host cells. The gold standard method for detection of NAbs is the plaque reduction neutralization test (PRNT). Based on the measurement of cell lysis due to viral infection, this test is able to detect antibodies that prevent cell infection (Muruato *et al*., 2020; Lau *et al*., 2021). This technique requires the use of live pathogens, *i.e.*, SARS-CoV-2 in this case, and must be done in a biosafety level 3 (BL3) laboratory. In addition, it requires expensive installations, skillful and meticulous staff, and a high workload, which prevents its wide implementation even in research laboratories. A SARS-CoV-2 pseudovirus will express the S protein responsible for cell entrance, but will not express the pathogenic genetic material of the virus, making them less dangerous for laboratory staff and the environment.

Keywords: COVID-19, SARS-CoV-2, Neutralizing antibody, Pseudo-type virus, Immune response

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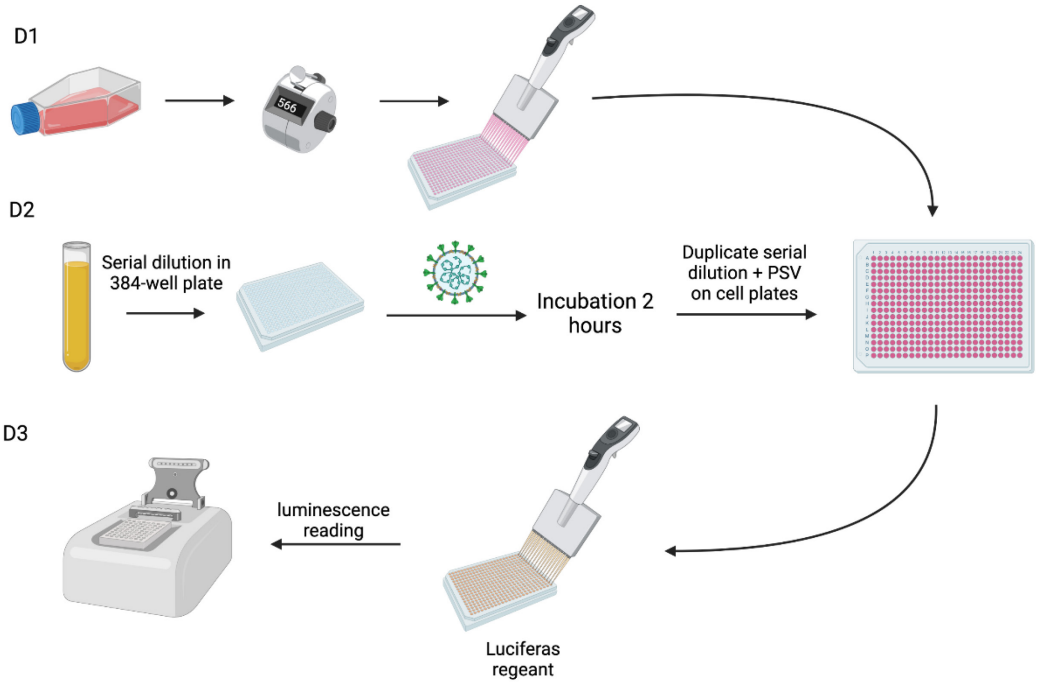
Graphic abstract:



Background

The gold standard method for detection of NAbs is the plaque reduction neutralization test (PRNT) (Perera *et al*., 2020). Based on the measurement of cell lysis due to viral infection, this test is able to detect antibodies that prevent the cell infection (Muruato *et al*., 2020; Lau *et al*., 2021). Such technique requires the use of live pathogens, *i.e.*, SARS-CoV-2 in this case, and must be done in a biosafety level 3 (BL3) laboratory. In addition, it requires expensive installations, skillful and meticulous staff, and a high workload, which prevent its wide implementation even in research laboratories (Muruato *et al*., 2020; Lee *et al*., 2021). Such facilities are not widely available, and only very specialized institutions can offer access to BL3 laboratories and trained staff. Quite similar neutralization techniques based on pseudoviral particles (called pseudo-virus neutralization tests, pVNT) have been developed, and can be performed in BL2 laboratories, allowing higher throughput (Nie *et al*., 2020a). A SARS-CoV-2 pseudovirus will express the S protein responsible for cell entrance, but will not express the pathogenic genetic material of the virus, making them less dangerous (Nie *et al*., 2020a, 2020b).

Materials and Reagents

1. Sterile white 384-well µClear flat bottom cell culture plate with lid (Greiner Bio-One, Kremsmünster, Austria, catalog number: 781098)
2. Sterile 384-well flat bottom assay plate with lid (Corning, NY, USA, catalog number: 3701)
3. Pipette tip 200 µL (Thermo Fisher Scientific, Waltham, MA, USA, catalog number: AM12650)
4. Eppendorf tube (Sigma-Aldrich, Saint-Louis, MO, USA, catalog number: T2795)
5. 50 mL reagent reservoir sterile polystyrene (Merck, Overijse, Belgium, catalog number: CLS4870)
6. HEK-293T hACE2 (Invivogen, San Diego, CA, USA, catalog number: HKB-hACE2)
7. SARS-CoV-2 Pseudoviral Particles (E-enzyme, Gaithersburg, MD, USA, catalog number: SCV2-PsV-001)

8. Dulbecco's Modified Eagle Medium (DMEM), with L-glutamine and glucose (Lonza, Bâle, Switzerland, catalog number: LO BE12-604F)
9. FireFly Luciferase kit (E-enzyme, Gaithersburg, MD, USA, catalog number: CA-L165-10)
10. Tryptan blue (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA, catalog number: T10282)

Equipment

1. Spectramax 3 iD (Molecular Devices, LLC, CA, USA)
2. Laminar flow hood (Thermo Fisher Scientific, Waltham, MA, USA, MSC Advantage 1.8 catalog number: 51025413)
3. Electronic multichannel 5–125 µL pipette (Brand, Transferpette -12 electronic, catalog number: 705453)
4. Monochannel 5–50 µL pipette (Socorex, Ecubens, Switzerland, catalog number: 825.0050)
5. Centrifuge 5702 (Eppendorf, Hamburg, Deutschland, catalog number: 5702000320)
6. Neubauer counting slide (Hecht Assistant, Altnau, Switzeland, catalog number: 40441)
7. Julobo ED Water bath (Sigma Aldrich, Saint-Louis, MO, USA, catalog number: Z615498)

Software

1. GraphPad Prism software (version 9.1.0, San Diego, CA, USA)

Procedure

A. Cell inoculation on a 384-well plate

To determine the quantity of cell suspension necessary, a calculation of this type must be made:

1. A volume of 15 µL of cell suspension within ± 8.5 × 10^3 cells are seeded in each well of the 384-wells plate. The quantity of cells suspension to prepare is 15 µL × 384 × X, where X is the number of plates to prepare.
2. To prepare this cell suspension, after counting and centrifugation, add the volume required to have 566 cells per µL.
3. To prepare the counting slide, the slat is stuck with water to the slide.
   a. I n an Eppendorf, add 50 µL of cell solution to put in the wells and 50 µL of trypan blue. Mix.
   b. Place 10 µL of this mix in each part of the counting slide. Under a microscope, the living cells inside the squares are counted. Living cells appear transparent and dead cells appear blue.
   c. To calculate the number of cells per milliliter, the following formula must be used, where n is the number of cells counted using a Neubauer counting slide.

$$\frac{Cells}{ml} = (n \times 10\,000)/(\frac{4 \times 1}{2})$$

4. The new cell suspension is maintained in a scotch bottle with constant agitation at moderate power. The 384-well plates are then filled with the cell suspension at a volume of 15 µL per well, with an **electronic multichannel micropipette**.
5. The plates are then annotated with "cell type—# of passages—operator's initials" and incubated in a calibrated oven for cell culture **at 37°C during 24 h**.

B. Serum dilutions

1. Heat inactivate the serums in a water bath **at 56°C for 30 min**.
2. Twenty-six sera can be diluted on a 384-well plate. Dilutions are made in line, and start at a 1:2 dilution, up to a 1:5120 dilution. If further dilutions are required, a second 384-well plate should be used.
3. Before making the serum dilutions, each well must be filled with 30 µL of dilution medium (DMEM + 10% HyClone FetalClone Serum) using **an electronic multichannel micropipette,** except for columns 2 and 12 which are filled with 50 µL.
4. Add 10 µL of serum in the first well, using a monochannel pipette 5–50 µL. Serial dilution of the sera can then be started, and proceeds as follows:
   - Flush 15 times in the aliquot.
   - Take a volume of 30 µL and place it in the first well.
   - Flush 15 times in the first well.
   - Change tips to collect liquid from the first to the second well.
   - Repeat the previous steps until the end of the serial dilution.

   In the last well, 30 µL must be removed, so that all wells contain the same volume.
5. A **cell control** (CC) and a **viral check** (VC) must be performed. The cell control (CC) is an assay in which **cells are incubated with culture medium**. The viral check consists of **the incubation of viruses without any sera**, in step C.1, 17.9 µL of SARS-CoV-2-PP must be incubated with 7.1 µL of culture medium.
6. Centrifugate at 161 × *g* during **5 min**.

C. Interaction between antibodies and Pseudoviral Particles

1. Dilute the pseudovirus three times in culture medium, to obtain the necessary volume for the analysis.
2. In each well of a 384-well plate, add 17.9 µL of diluted SARS-CoV-2-Pseudoviral Particles with an **electronic multichannel micropipette,** and 7.1 µL of dilution serums previously perfomed with a **manual multichannel micropipette (5 µL–50 µL)**. Each sample is carried out in duplicate. For one dilution serum, two tests are carried out.
3. The plates are then annotated with "operator's initial" and incubated in a calibrated oven for cell culture **at 37°C during 2 h**.

D. Innoculation of the virus on cells

1. First, start by emptying the culture medium from the 384-well plate containing the cells. Once this is done, transfer 17.5 µL of each column from the plate containing serum dilutions and virals particles to the cells palte with a **manual multichannel micropipette (5 µL–50 µL)**. Repeat the procedure, changing tips between each serum.
2. Add 7.5 µL of DMEM + 10% FC into each well.
3. Let incubate for **42 h at 37°C**.

E. Signal Measurement

1. Remove the supernatant and add 20 µL of eEnzyme's luciferase assay reagent into each well with an **electronic multichannel micropipette**.
2. Read in a luminescence plate reader. There must be a proportional relationship between luminescence and dilutions, the higher the dilutions, the higher the signal. Indeed, the luciferase enables the detection of infected cells, the more there is of antibody, the less the cells will be infected.

Data analysis

Based on the relative light units (RLU) values from each sample, a percentage of inhibition can be calculated. The following formula must be applied to each dilution for each sample:

$$Relative\ inhibition = \frac{RLU\ sample\ X - RLU\ negative\ control}{RLU\ viral\ control - RLU\ cell\ control}$$

The different percentages of inhibition are used to plot the evolution of the relative inhibition as a function of the serum dilution. By intrapolation of the sigmoid curves obtained, it is possible to determine the dilution at which 50% inhibition is achieved, called the RI50. The results obtained via statistical software give us the logarithm of the dilution in comparison to the 1:10 dilution, considered our initial condition. This logarithm is then transformed into a numerical dilution within the range achieved. A sample is considered negative if the RI50 value of this sample is below the 1:20 dilution. An example of the expected results is shown in Figure 1.

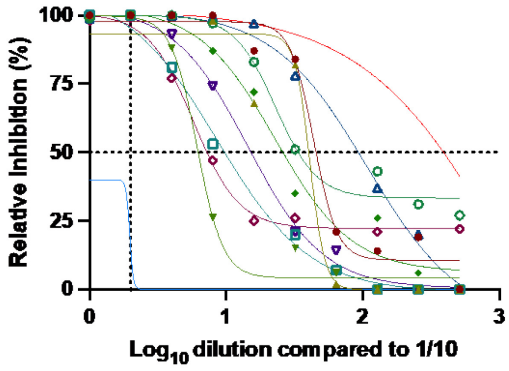


Figure 1. Percentage of relative inhibition as a function of the log_{10} of the dilution compared to the 1:10 dilution.

Acknowledgments

We acknowledge Nie *et al*. (2020b) for the paper "Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay" in Nature Protocol, our protocol was direved from.

Competing interests

All authors have none to declare.

Ethics

The protocol was in accordance with the Declaration of Helsinkiand was approved by the Medical Ethical Committee of Saint-Luc Bouge (Bouge, Belgium,approval number B0392020000005). The informed consent was obtained from all subject.

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