Apr 09, 2025

Multiplex surrogate Virus Neutralization Test (sVNT)

DOI
https://dx.doi.org/10.17504/protocols.io.4r3l2qkm4l1y/v1

Javi Adividya[1], Baiq Ulfana Syabila[1], Jessica Ferinstyadewi Gunawan[1], Mesyalie Lorenza[1], Adriel Osmandya Hanari[1], Alnafisya Syamsu[1], Anuraj H. Shankar[1]

[1]Summit Institute for Development

J Javi Adividya
Summit Institute for Development

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.4r3l2qkm4l1y/v1

Protocol Citation: Javi Adividya, Baiq Ulfana Syabila, Jessica Ferinstyadewi Gunawan, Mesyalie Lorenza, Adriel Osmandya Hanari, Alnafisya Syamsu, Anuraj H. Shankar 2025. Multiplex surrogate Virus Neutralization Test (sVNT). **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l2qkm4l1y/v1

Protocol status: Working
We use this protocol and it's working

Created: September 24, 2024

Last Modified: April 09, 2025

Protocol Integer ID: 108291

Keywords: sVNT, Luminex, surrogate Virus Neutralization Test, surrogate virus neutralization test, neutralizing antibody, neutralizing antibody response, neutralization assay, specific antibodies to color, using biotinylated antibody, simultaneous detection of multiple analyte, biotinylated antibody, specific antibody, antibody response, luminex method, evaluating protective immunity, luminex xmap technology, multiple analyte, scalable alternative to traditional virus, luminex instrument measure, traditional neutralization test, traditional virus, luminex, assay, live virus, protective immunity, coupling analyte, virus, coded microsphere

Abstract

The Surrogate Virus Neutralization Test (sVNT) integrated with Luminex xMAP technology provides a rapid, efficient, and safe method for assessing neutralizing antibody responses. Unlike traditional neutralization tests (VNT and pVNT), which require live viruses and cells, sVNT uses a cell-free approach, significantly reducing biosafety risks and processing time. Luminex's bead-based platform allows simultaneous detection of multiple analytes, including neutralizing antibodies, in a single sample. The test involves coupling analyte-specific antibodies to color-coded microspheres, followed by detection using biotinylated antibodies and a streptavidin-phycoerythrin (PE) reporter. A Luminex instrument measures the binding and neutralizing activity through laser-based detection. The sVNT-Luminex method offers a highly efficient workflow, requiring just 2 to 3 hours and basic lab equipment, making it accessible for use in BSL2 laboratories. This approach delivers high-throughput, multiplexed analysis, providing a practical and scalable alternative to traditional virus-based neutralization assays for evaluating protective immunity.

Guidelines

The Surrogate Virus Neutralization Test (sVNT) using Luminex xMAP technology involves several key steps for detecting neutralizing antibodies, particularly for COVID-19 research. First, samples such as serum or plasma are prepared by diluting them to the appropriate concentration, along with the preparation of standards and controls. Color-coded Luminex microspheres are coupled with specific antibodies, such as those targeting the SARS-CoV-2 spike protein, with each microsphere population representing a distinct analyte. These antibody-coupled beads are incubated with the samples to allow neutralizing antibodies to bind. After washing away unbound components, a PE-conjugated ACE2 receptor is added, which binds to any remaining unblocked spike proteins, providing a measure of inhibition. The streptavidin-phycoerythrin (PE) reporter is then added to detect the ACE2 bound to the microspheres. Data acquisition is performed using a Luminex instrument, where one laser identifies the specific bead region and another measures the PE signal, which correlates with the neutralizing activity of the sample. Results are analyzed by comparing the PE signal to a standard curve, allowing for the quantification of neutralizing antibodies based on the percentage inhibition of the ACE2-spike protein interaction. Proper calibration of the Luminex instrument, correct sample preparation, and adherence to biosafety guidelines are essential to ensure reliable results.

Materials

- Sodium chloride **P212121**
- 1X PBS (Phosphate-buffered saline)
- Bovine Serum Albumin (BSA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A7906
- PE-conjugated human ACE2 **Genscript**

Safety warnings

- **Biosafety Compliance**: Always perform the assay in a BSL2 laboratory to ensure proper containment and safety when handling human samples.
- **Cross-Contamination**: Avoid cross-contamination by thoroughly cleaning pipettes, using new tips for each sample, and following strict aseptic techniques.
- **Instrument Calibration**: Ensure the Luminex instrument is properly calibrated before running assays to avoid inaccurate results.
- **Reagent Stability**: Use freshly prepared or properly stored reagents to ensure optimal performance. Expired or improperly stored reagents can lead to inconsistent data.
- **Sample Integrity**: Handle samples carefully to avoid degradation. Improper handling or storage may affect the accuracy of the results.

Before start

- **Prepare Reagents**: Have all necessary reagents, including color-coded microspheres, biotinylated antibodies, PE-conjugated ACE2 receptor, and wash buffers ready.
- **Sample Preparation**: Dilute serum or plasma samples to the correct concentration. Prepare controls and standards for reference.
- **Biosafety Measures**: Conduct the assay in a BSL2 lab and follow standard safety protocols for handling human samples.

Reagent Preparation

30s

1 Prepare for **Assay Buffer A**

2d

Note

100 mL 1x PBS solution
1 g Bovine Serum Albumin (BSA)
5.85 g NaCl

- To prepare 100 mL of **Assay Buffer A**, dissolve 5.85 g of NaCl to make a [M] 1 Molarity (M) NaCl solution in 100 mL sterile 1x PBS solution.
- Mix the [M] 1 Molarity (M) NaCl solution with 1 g of BSA to make a final solution of [M] 1 Mass / % volume BSA concentration.
- Stir the solution until the BSA completely dissolved.

Note: **Assay Buffer should not be re-used after** 48:00:00 **, please prepare a fresh batch of assay buffer before every run of sVNT.**

2 Prepare for **Assay Buffer B**

Note

100 mL 1x PBS solution
1 g Bovine Serum Albumin (BSA)

- To prepare 100 mL of **Assay Buffer B**, dissolve 1 g of BSA to make a [M] 1 Mass / % volume BSA solution in 100 mL sterile 1x PBS solution.
- Stir the solution until the BSA completely dissolved.

3 Prepare for **PE-conjugated human ACE2**

Note

4989.5 µL Assay Buffer A

[M] 950 Mass Percent PE-conjugated human ACE2

To perform this assay, we need to make a PE-ACE2 working solution with a final concentration of 2000 ng/mL. The concentration of the stock reagent of PE-ACE2 is 950 ug/mL. So to make a PE-ACE2 working solution for 1 plate (96 well with 50 µL per reaction) , by taking pipetting error into account we will prepare for 100 reactions with a total volume of 100 x 50 µL = 5000 µL of working solution.

- Dilute 10.5 µL of PE-conjugated ACE2 in 4989.5 µL of **Assay buffer A** in a 15 mL conical tube.
- Mix well by inverting the tube several times.

4 Prepare the **RBD-conjugated MagPlex® beads**

Note

15 µL of each RBD-conjugated MagPlex beads
2275 µL Assay Buffer A

To perform this assay, we need 600 beads per reaction (per well). The concentration of stock reagent of RBD-conjugated MagPlex bead is [M] 4000 beads/uL . The formula for making the MagPlex beads for 1 plate (96 reactions), with added reactions to take into account pipetting error (100 reactions) is shown below:

Firstly, we need to calculate the total beads needed for 1 plate or 100 reactions:
(100 reactions x 600 beads) : [M] 4000 beads/uL = 15 µL

Then we calculate the total volume solution needed for 1 plate or 100 reactions:
(100 reactions x 25 µL) = 2500 µL .

Since we have in total 15 types of beads, we need to calculate the total volume of MagPlex beads we use to adjust the volume of solvent (**Assay buffer A**) we need:
(15 x 15 µL) = 225 µL .

Finally, we calculate the volume of solvent we need:
2500 µL – 225 µL = 2275 µL .

- To prepare the beads, pool 15 µL of each beads in a 5 mL tube.
- Dilute the pooled beads with 2275 µL of **Assay buffer A** making a total solution of 2500 µL .
- Mix well by inverting the tube several times.

Note: **Beads should not be exposed to light as much as possible and should be kept at 4 °C for storage. When reconstituting beads in buffer, each tube should be vortexed for ~40 seconds before use.**

5 Prepare for test sera/plasma sample by thawing it at Room temperature . Then vortex the sera/plasma for 00:00:15 , followed by spin down at 6000 rpm, 00:00:15 . 30s

Procedure 1h

6 In a 96-well plate, dilute 4 µL of test sera/plasma into a well, containing 36 µL of **Assay buffer A** for a final dilution of 1:10.

7 Continue the serial dilution to make a concentration of 1:40 by transferring 1 µL of the diluted plasma into another well containing 39 µL of **Assay buffer A**.

8 Repeat the step above 2 more times to make 4 titer concentration in total (1:10, 1:40, 1:160, 1:640)

9 In a new 96-well plate, add 25 µL of the diluted RBD-conjugated MagPlex beads into each well.

10 Transfer 25 µL of the diluted sera/plasma to it's corresponding well.

11 Add 25 µL of the positive and negative control separately that has been diluted previously with a dilution ratio of 1:10.

Note

Please prepare a duplicate for both the positive and negative control. This will take up 4 wells in the plate, so please keep this in mind when making the mapping of the plate.

12 Seal the plate with an adhesive plate sealer.

13 Incubate the plate on a plate shaker for 200 rpm, 01:00:00 at 24 °C .

14 After the incubation is done, take the plate off of the shaker then peel off the adhesive sealer carefully as to not spill any liquid from the wells.

15 Then add 50 µL of the [M] 2000 ng/mL PE-conjugated human ACE2 into each well.

16 Seal the plate again with an adhesive plate sealer

17 Incubate the plate for a second time on a plate shaker for 200 rpm, 01:00:00 at 24 °C .

18 After the incubation is done, take the plate off of the shaker then peel off the adhesive sealer carefully as to not spill any liquid from the wells.

19 Place the plate on a plate magnetic rack for 00:03:00 to collect all the beads to the bottom of the well. 3m

20 Dump the solution carefully by flicking the plate once with the magnetic rack attached as to not discard the beads.

21 Add 100 µL of **Assay buffer A** to each well.

22 Allow the plate to incubate for 00:02:00 at Room temperature . 2m

23 Discard the solution without removing the plate from the magnet.

24 Repeat step 20 - 23 once.

25 Then add 120 µL of **Assay Buffer B** ([M] 1 Mass / % volume BSA in 1X PBS solution) to each well.

26 Place the plate on a plate shaker, then shake the plate at 24 °C for 800 rpm, 00:02:00 .

27 Read plate on the Luminex

Data Analysis

28 For analyzing the results, you will get the Excel file results consisting of several different data, including Net MFI and Count data. We will use the Net MFI and Count data to analyze the results. First, you need to identify the position of each sample by renaming the column (Fig. 1)

(A).

(B).

Fig. 1. The before (A) and after (B) renaming the sample name.

29 After renaming the samples, you also need to identify which one(s) is the positive and negative control. This is important later on during the data calculation.

30 Then, we will calculate the %inhibition using the Net MFI value of the sample (Fig. 2). The %inhibition will be displayed as a **percentage**. So, when you got your data as 97.83254 it means it is 97,8%. The higher the %inhibition, the higher the inhibiting ability of the sample's antibody, meaning that the neutralization reaction is higher.

Fig. 2. Equation for calculating the %inhibition

31 Make a new table to ease the calculation (Fig. 3). We will make the list of antigens the same as the list on the Net MFI table. The list here indicates the antigens that were detected during the reading process.

Fig. 3. Table for calculating the %inhibition. The first row of this table (SARS-1, XBB1.5, etc) follows the first row on the Net MFI table.

32 To calculate the data, we will select the negative control on row 74 as the negative control base, that will be used in the formula. You can choose either negative control. Now, to calculate the IC of SARS-1 on sample H-01, we can put the formula like this: **=(1-(C61/C74))*100**. C61 here indicates the Net MFI data for sample H-01 on antigen SARS-1, while the C74 here indicates the negative control for SARS-1 that we chose previously. Please make sure to use **$** (absolute references) so that the cell C74 won't change if you drag the formula. Continue doing so until the Mu antigen or the last antigen is done. The results should look like (Fig. 4).

Fig. 4. Result example

Note

The value for negative control that we chose should be **zero**, as it was divided by itself on the formula. While the value for both positive controls should be **within 98% to 99%.** If you encounter a positive control that shows a value below 93%, it may indicate that the assay is not optimally run, which may indicate that either the positive control has been degraded or the positive control is missing from the reaction (not added).

33 Then convert the %inhibition data into IC50 using the data of all 4 dilutions from each sample by using this python code.

Command

This python code can be used to calculate IC50.

ic50.py

```
import pandas as pd
from py50.calculator import Calculator
from py50.plotcurve import PlotCurve
from py50.plot_settings import CBMARKERS, CBPALETTE
import matplotlib.pyplot as plt

dataset_path = 'your_file.csv'

data = pd.read_csv(dataset_path)

calc_data = Calculator(data)

calc_data.show()

calculation_absolute_ic50 = calc_data.calculate_absolute_ic50(
    name_col='sample',            # Column for sample/compound name
    concentration_col='dilution', # Column for dilution/concentration
    response_col='response'       # Column for response (e.g., %
inhibition)
)

print(calculation_absolute_ic50)

calculation_pic50 = calc_data.calculate_pic50(
    name_col='sample',            # Column for sample/compound name
    concentration_col='dilution', # Column for dilution/concentration
    response_col='response'       # Column for response (e.g., %
inhibition)
)

print(calculation_pic50)

calculation_pic50.to_csv('pic50_results.csv', index=False)

plot = PlotCurve(data)
plot.plot(
    name_col='sample',            # Column for sample/compound name
    concentration col='dilution'. # Column for dilution/concentration
```

```
    response_col='response',      # Column for response (e.g., %
inhibition)
    markers=CBMARKERS,            # Use custom markers
    palette=CBPALETTE             # Use custom color palette
)

plt.show()
```

Protocol references

1. RnD Systems. (n.d.). What is a luminex® assay?. (Please find the article **here**)
2. Tan, C. W., Chia, W. N., Qin, X., Liu, P., Chen, M. I.-C., Tiu, C., Hu, Z., Chen, V. C.-W., Young, B. E., Sia, W. R., Tan, Y.-J., Foo, R., Yi, Y., Lye, D. C., Anderson, D. E., & Wang, L.-F. (2020). A sars-COV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2–spike protein–protein interaction. Nature Biotechnology, 38(9), 1073–1078. https://doi.org/10.1038/s41587-020-0631-z (Please find the article **here**)