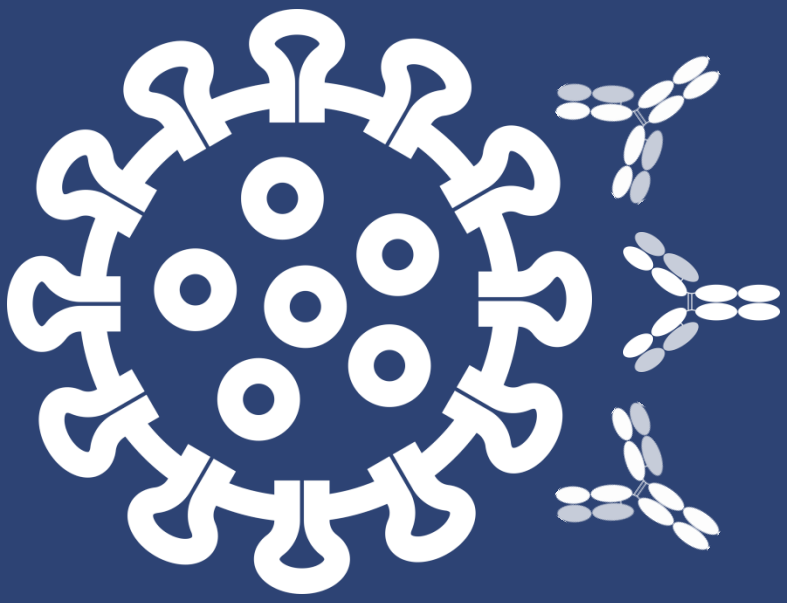

ProteoGenix

Technical Guide and Protocol

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit

Catalog number KPTX02

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INTRODUCTION

SARS-CoV-2, the etiological agent of the COVID-19 disease, is a pandemic strain of Coronavirus with presumable zoonotic origins. The new strain contains several structural proteins such as the spike glycoprotein (S), envelope (E), membrane (M), and the nucleocapside (N). The strain also contains 16 additional non-structural proteins (Nsp) with different roles in viral infection and replication.

The infection initiates when SARS-CoV-2 binds the human ACE2 receptor (angiotensin-converting enzyme 2) through the receptor-binding domain (RBD) present in the *N*-terminal of the spike glycoprotein. One way to arrest the infectious cycle is to block RBD-ACE2 interaction with neutralizing antibodies.

But due the high virulence of the SARS-CoV-2 strain, viral neutralization tests can only be performed through time-consuming and technically-demanding approaches such as pseudo-virus neutralization assays (pVNT) or in facilities with a strict biosafety containment (BSL3) such as plaque reduction neutralization tests (PRNT).

sVNT TEST PRINCIPLE

Designed in the versatile ELISA format, the sVNT assay allows the quick screening of SARS-CoV-2 neutralizing activity of candidate antibodies without the need to use native viral particles nor mammalian cells. It is compatible with purified antibodies from any species, format, or isotype and it serves to accelerate the development of anti-SARS-CoV-2 antibodies for therapy, diagnostics, and research.

The assay works according to the principle of competitive binding between a purified antibody and horseradish peroxidase labeled ACE2 for the binding pocket of SARS-CoV-2 RBD **[Figure 1]**. Since the label is on the human ACE2, the neutralizing activity of an antibody (Nab) will be inversely proportional to the test signal ($OD_{450\ nm}$).

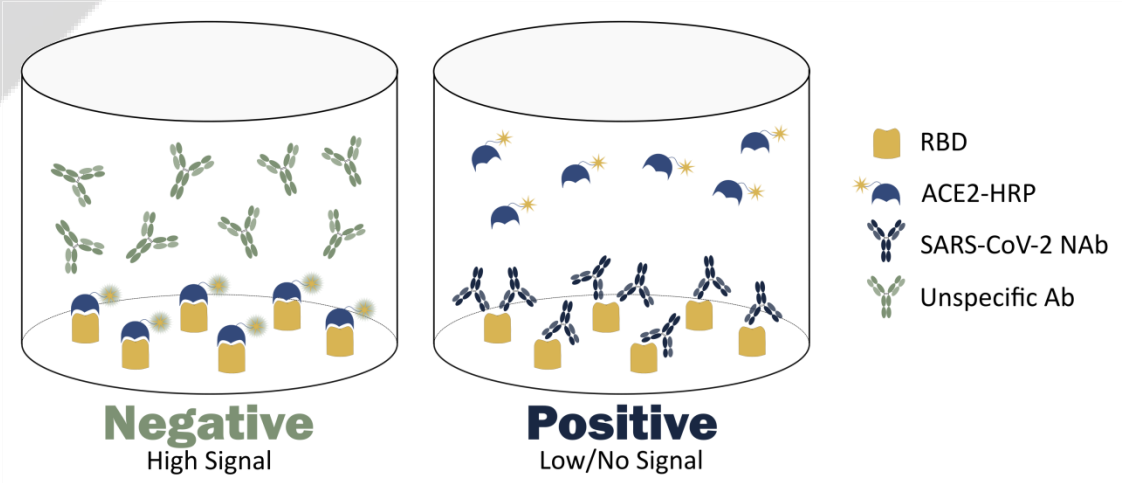

Figure 1. Schematic representation of the **sVNT** principle of detection in ELISA format.

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PRODUCT COMPONENTS

Each sVNT assay kit includes the following components:

COMPONENT	QUANTITY/SIZE
Capture Plate	1 plate (96 wells)
Positive Control	12 µl (1 vial), 1mg/mL
Negative Control	60 µl (1 vial)
Detection A: HRP Conjugated ACE2	60 µl (1 vial)
Reagent Dilution Buffer	25 ml (1 vial)
Sample Dilution Buffer	25 ml (1 vial)
20x Wash Solution	25 ml (1 vial)
TMB Solution	12 ml (1 vial)
Stop Solution	6 ml (1 vial)
Plate Sealer	4 pieces

PRODUCT DETAILS

Kit Size	96 tests
Sample Type	Purified Antibody
Instrument	Colorimetric Microplate Reader
Measurements	$OD_{450\ nm}$
Shipping Conditions	Blue Ice
Storage Conditions	Stable for 6 months from the date of manufacture, if Capture Plate, Controls and HRP conjugated ACE2 are kept at -20°C. The remaining reagents can be stored at 4°C.
Assay Kit Format	Competitive ELISA
Assay Type	Qualitative
Label or Dye	Horseradish Peroxidase (HRP)
Substrate	3,3′,5,5′-Tetramethylbenzidine (TMB)
Validation data	Tested with purified antibodies (neutralizing and non-neutralizing) and convalescent/healthy human serum

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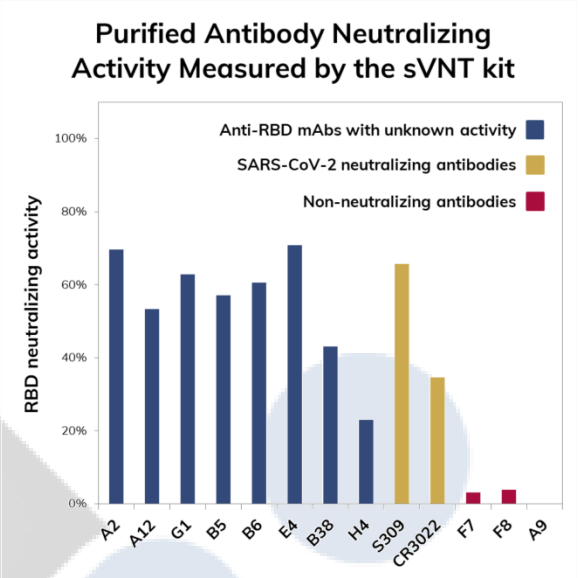
RECOMMENDATIONS FOR PURIFIED ANTIBODIES



These tests were performed with **1 µg/ml** of each purified antibody under the standard protocol.

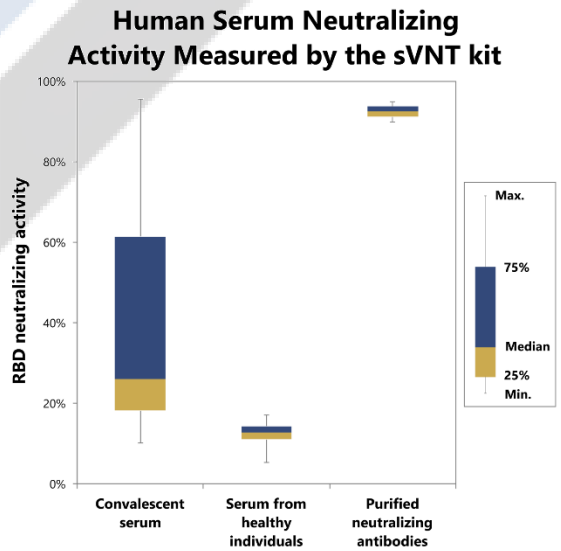
Antibodies **S309** (doi: 10.1038/s41586-020-2349-y) and **CR3022** (doi: 10.1038/s41467-020-16256-y), shown to neutralize SARS-CoV-2 in conventional PRNT assays, were used as positive controls.

Non-neutralizing antibodies were shown to bind to the S2 subunit of SARS-CoV-2 and thus used as **negative controls**.

Anti-RBD antibodies isolated from the **Covid-19 human antibody library** and with confirmed RBD-binding specificity using ELISA and Biacore SPR, served as test samples.

Results indicate that the inhibition ratio (=neutralizing activity) obtained through the sVNT kit correlates strongly with an antibody's potential for neutralizing the SARS-CoV-2 virus.

RECOMMENDATIONS FOR SERUM SAMPLES



Convalescent serum (n = 14), 10-fold diluted, was used to monitor the development of neutralizing activity in patients and compared to samples from healthy individuals and purified antibodies with proven neutralizing activity.

But while a basal signal was detected in the serum from healthy individuals (second box on the boxplot), **the inhibition ratios were significantly inferior** to the ones detected in convalescent serum samples.

These **results are consistent with previous reports** of the variability found in the immune response of different individuals (symptomatic and asymptomatic) to the SARS-CoV-2 virus (doi: 10.1038/s41591-020-0965-6).

Results showed a strong correlation between exposure to SARS-CoV-2 virus and the development of a measurable neutralizing immune response in some patients.

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SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines.

Handle all blood and serum samples according National Committee for Clinical Laboratory Standards (NCCLS) recommendations for handling and storing these samples (Approved Standard-Procedures for the Handling and Processing of Blood Specimens, H18-A. 1990).

SERUM ▶ Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x *g*. Collect serum and test it immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles.

PLASMA ▶ Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x *g* within 30 minutes of collection. Test plasma samples immediately or aliquot and store them at ≤ -20℃. Avoid repeated freeze-thaw cycles.

INTERPRETATION OF RESULTS

To ensure assay validity, each assay must include both a Positive and Negative controls with OD_{450nm} values within the range listed in table below. If values do not meet the requirements, the test must be considered invalid and repeated.

Control	Acceptable OD_{450nm} values	Interpretation
Positive	< 0.3	Passed
Negative	> 0.9	Passed

The estimation of SARS-CoV-2 inhibition rates (%) and cutoff values for interpretation can be found in the formula and table below:

$$Inibition\ rate(\%) = \left(1 - \frac{OD_{450\ sample}}{OD_{450\ negative\ control}}\right) \text{x } 100$$

Inhibition rate (%) cutoff	Result	Interpretation
≥ 20%	Positive	Neutralizing activity detected
< 20%	Negative	Negligible neutralizing activity

Intra-Assay Precision (Precision within an assay): < 8%
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays): < 10%
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision.

TEST PREPARATION

REAGENT PREPARATION

1. Take all reagents from cold storage (-20 or 4 °C) and allow them to return to room temperature before use (20 to 25°C). Store all reagents at the recommended storage temperature promptly after use.
2. Vortex all reagents before use.
3. **Detection A working solution** preparation procedure: dilute **HRP-ACE2** in **Reagent Dilution Buffer** in a 1:200 volume ratio.
4. 1× Wash Solution preparation procedure: dilute the **20× Wash Solution** in deionized or distilled water in a 1:20 volume ratio. Store the solution at 2-8°C when not in use. *Example: to prepare 400 ml of this solution dilute 20 ml of 20× Wash Solution in 380 ml deionized or distilled water.*

SAMPLE AND CONTROL PREPARATION

5. Take all samples and controls from cold storage (-20 or 4 °C) and allow them to return to room temperature before use (20 to 25°C). Store them back at the recommended storage temperature promptly after use.
6. Vortex all samples and controls before use.
7. Positive and Negative Control preparation procedure: dilute positive control with the **Sample Dilution Buffer** in a 1:100 volume ratio. *Example: to prepare 500 µl of the control solution dilute 5 µl of each control in 495 µl of Sample Dilution Buffer.* Dilute negative control with the **Sample Dilution Buffer** in a 1:10 volume ratio.
8. Sample preparation procedure: dilute samples with the **Sample Dilution Buffer** in a 1:10 volume ratio (this may need to be optimized according to the concentration of your sample). *Example: to prepare 100 µl dilute 10 µl of each sample in 90 µl Sample Dilution Buffer.*

CAPTURE PLATE PREPARATION

CAPTURE PLATE PREPARATION

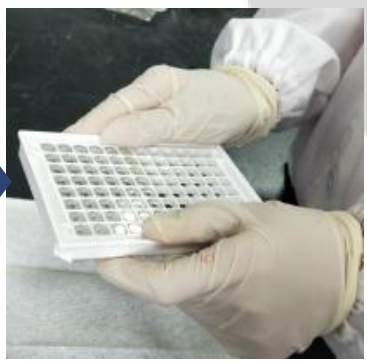
For more accurate results it is recommended to measure each sample and control in duplicate.

9. Prepare the number of strips according to the number of samples you will be measuring. Install the strips making sure they are tightly snapped to the plate frame (see detailed protocol bellow).
10. Keep the unused strips in the closed foil pouch (to prevent damage from moisture) and store them at -20 °C.

INSTALL AND REMOVE STRIPS FROM THE PLATE

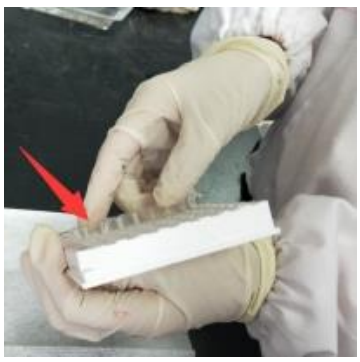
STEP 1

Hold the plate containing the pre-installed strips



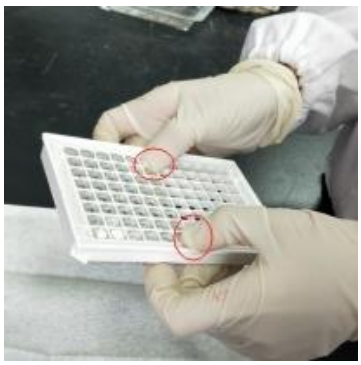
STEP 2

Carefully turn it over



STEP 4

Remove the loosen strip from the frame



STEP 3

Press opposite wells A and H from the same strip

NEUTRALIZATION REACTION

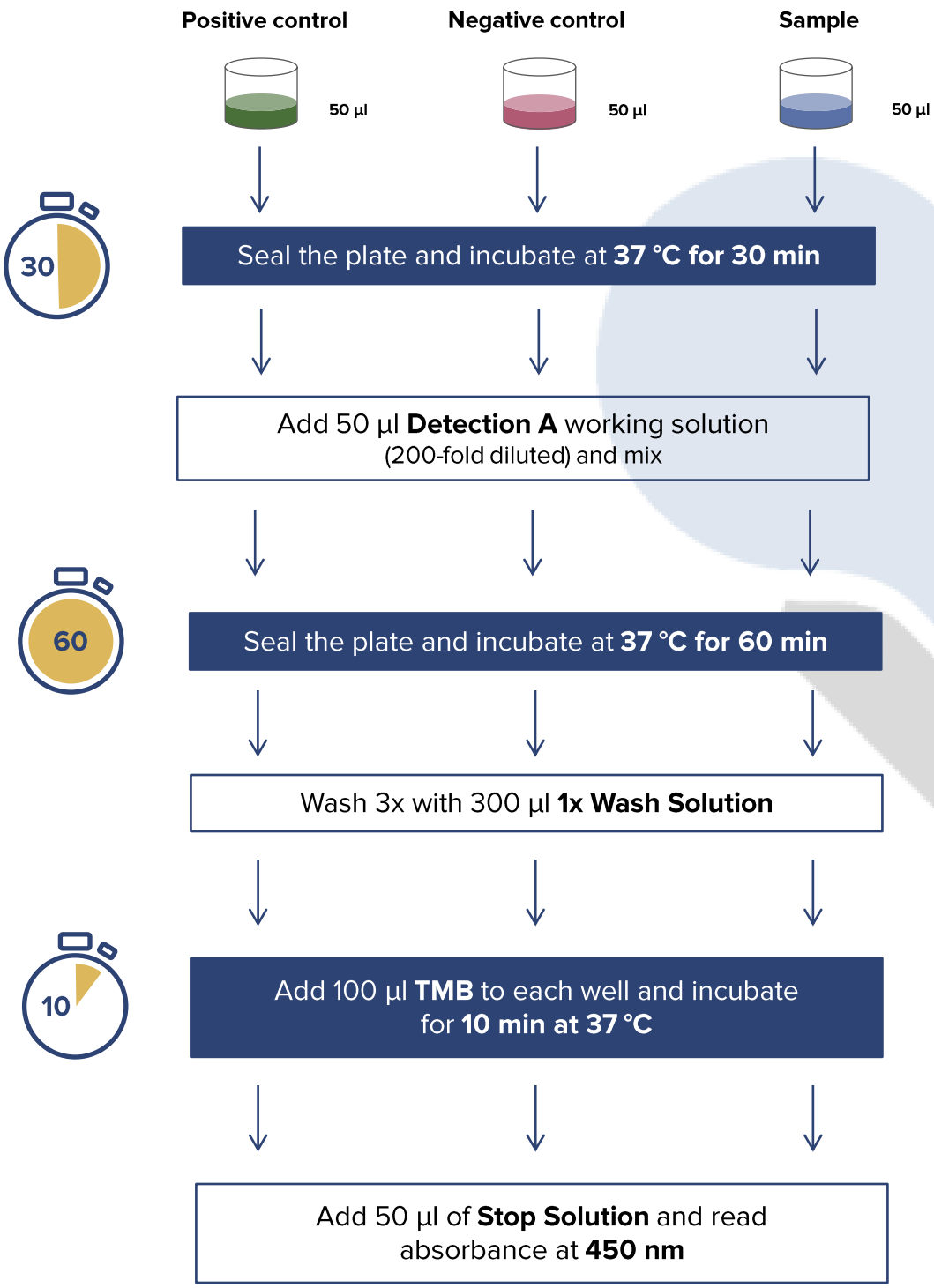
11. In separate wells, add 50 µL of the 100-fold diluted **Positive Control**, 100-fold diluted **Negative Control**, and the **Samples**.
12. Cover the **Capture Plate** with the **Plate Sealer** and incubate at **37 °C for 30 minutes**.
13. Add 50 µL of diluted **Detection A working** solution to each well. Set the plate on the microplate mixer and shake **for 5 minutes**.
14. Cover the **Capture Plate** with the **Plate Sealer** and incubate at **37 °C for 60 minutes**.
15. Remove the **Plate Sealer** and wash the plate with 300 µl **1× Wash Solution** using a squirt bottle, manifold dispenser, or autowasher. Remove any residual liquid by inverting the plate and placing it over a clean paper towel to absorb residual droplets.
16. Repeat step (15) 3 times.
17. Pat the plate on a clean paper towel to remove residual liquid in the wells after washing.

SUBSTRATE REACTION AND MEASUREMENT

17. Add 100 µl of **TMB Solution** to each well and incubate the plate in the dark at **37 °C for 10 minutes** (start timing after the addition of TMB Solution to the first well).
18. Add 50 µl of **Stop Solution** to each well to quench the reaction. The color in the wells should change from blue to yellow. If the color is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
19. Read the absorbance in a microplate reader at 450 nm within 10 minutes. If wavelength correction is available, set it to 540 nm or 570 nm. If not, subtract readings at 540 nm or 570 nm from the readings at 450 nm to correct optical imperfections in the plate.

*Note: Substrate reaction time is determined by the reaction temperature. The ideal reaction temperature is **37 °C**. If the temperature is lower than 37 °C, extend the reaction time appropriately.*

SIMPLIFIED PROTOCOL



TROUBLESHOOTING GUIDE

Problem	Possible cause	Solutions
Positive control signal is weak or abnormal	Detection A reagent and pre-coated plates possible activity loss	a. Increase the reaction time or the concentration of the Detection A reagent to compensate for possible loss of protein activity
	Errors in instrument settings	a. Check instrument settings b. Start the ELISA detecting instrument at least half an hour before use
	Pipetting errors	a. Make sure that your pipette is calibrated and working properly
	Detection A solution prepared too far in advance	a. Repeat the assay with freshly prepared Detection A working solution
	Temperature fluctuations	a. Use proper incubation time and temperature and avoid repeated freeze-thaw cycles
High background signal	Sample solvent contains inhibiting factors	a. Include a blank (solvent alone) in your assays b. Maintain DMSO concentrations at < 1%
	Insufficient washing	a. Increase number of washes and the volume Wash Buffer used
	Buffer or sample contamination or degradation	a. Make sure buffers and samples are prepared, used, and stored correctly
Erratic colorimetric signal	Inconsistent pipetting or dilution methods	a. Make sure pipettes are working properly and use a multichannel pipette whenever possible to reduce pipetting inconsistencies b. Prepare master mix solutions to minimize errors c. Test in duplicate or triplicate for more accurate results
	Presence of bubbles in the wells	a. Tap the plate gently to disperse bubbles
	High signal intensity	a. The concentration of the samples should be adjusted to achieve optimal signal intensity b. Decrease mixture incubation time to 45 min
	Incomplete mixing of Detection A and sample	a. Make sure that the Detection A reagent is completely mixed with the sample
Inadequate color development	Residual buffer or contamination in the wells	a. Color in the negative control should appear within 10 seconds after the TMB substrate is added. If this is not the case: a. Ensure no contamination or residual buffers are left in the wells before adding TMB b. Increase TMB incubation time to 15 min