SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit

Base Catalog # D-1008

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit is designed for detecting and quantifying SARS-CoV-2 neutralizing antibody in circulation in a fast and high throughput format.

Input Material: Input materials can be serum, plasma, and various body fluids.

Internal Control: A positive control (SARS-CoV-2 neutralizing antibody) and a negative control (non-immune IgG) are provided in this kit for the quantification of sample neutralizing antibody. Because SARS-CoV-2 neutralizing antibody in the samples can vary with different sample source, and from different diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

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KIT CONTENTS

Component	48 Assays Cat. #D-1008-48	96 Assays Cat. #D-1008-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
AB (Assay Buffer)	6 ml	12 ml	RT
PC (Positive Control, 1 mg /ml)*	5 µl	10 µl	4°C
NC (Negative Control)*	4 µl	8 µl	4°C
ACE2 (His-ACE2 Protein, 1 mg/ml)*	8 µl	16 µl	–20°C
NDC (Neutralizing Detection Complex, 2000X)*	4 µl	8 µl	–20°C
DS (Developer Solution)	5 ml	10 ml	4°C
SS (Stop Solution)	5 ml	10 ml	RT
8-Well Assay Strips (With Frame)	6	12	4°C

**Spin the solution down to the bottom prior to use.*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C.

Upon receipt: (1) Store **ACE2**, and **NDC** at -20°C away from light; (2) Store **WB**, **PC, NC, DS** and **8-well assay strips** at 4°C away from light; (3) Store all remaining components (**AB** and **SS**) at room temperature away from light.

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Note: *(1) Check if **WB** (10X Wash Buffer) contains salt precipitates before use. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved. (2) Transfer the amount of **DS** (Developer Solution) required into a secondary container (tube or vial) before adding **DS** into the assay wells in order to avoid contamination. Check if a blue color is present in **DS** before each use, as this would indicate contamination of the solution and should not be used.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette or multiple-channel pipette
- ☐ Multiple-channel pipette reservoirs
- ☐ Aerosol resistant pipette tips
- ☐ Microplate reader capable of reading absorbance at 450 nm
- ☐ 1.5 ml microcentrifuge tubes
- ☐ Incubator for 37°C incubation
- ☐ Distilled water

- ☐ Sample of interest
- ☐ Parafilm M or aluminium foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of this product is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: This product is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: This product and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

COVID-19 is an infectious disease caused by SARS-CoV-2, a new member of the same coronavirus family that caused SARS and MERS. Entry of SARS-CoV-2 into human host cells occurs through binding of surface unit S1 of its spike protein to the cell receptor angiotensin-converting enzyme 2 (ACE2), which leads to endocytosis, viral replication, and then spreads SARS-CoV-2 infection [1,2]. Such infection typically induces antibody response. The antibody is able to bind to the SARS-CoV-2 spike, and only a small part of the antibody is with neutralizing function. Only neutralizing antibody is capable of blocking the action of SARS-CoV-2 spike, and inhibits the SARS-CoV-2 virus to infect new cells [3].

It is critically important to detect SARS-CoV-2 neutralizing antibody in a quantitative and high throughput format. (1) It can help to identify whether the infected individuals with or without symptoms have acquired protective immunity from COVID-19 and how long the neutralizing antibodies persist after infection; (2) it can help to accurately evaluate the therapeutic antibodies against SARS-CoV-2; (3) It would greatly benefit the development of the effective vaccine.

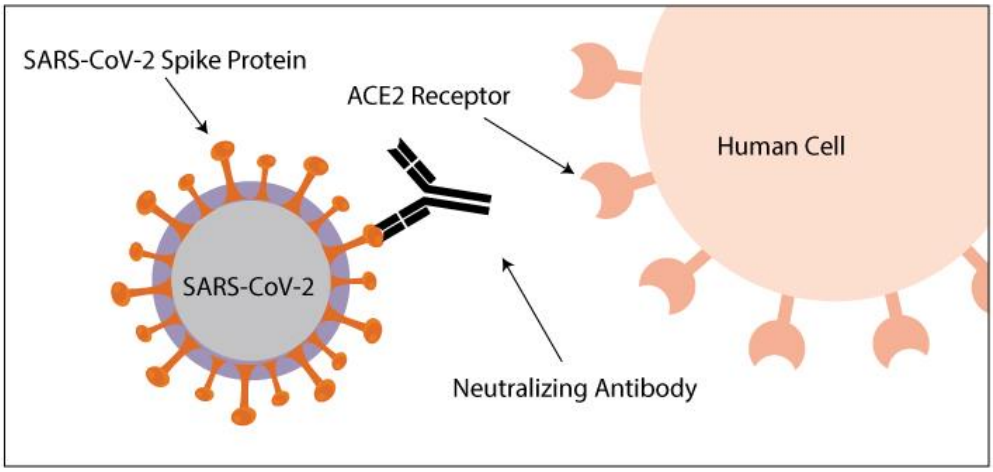

Fig 1. Inhibition of SARS-Cov-2 by neutralizing antibody.

There are several methods used for measuring SARS-CoV-2 neutralizing antibody, which include plaque reduction neutralizing test (PRNT), virus neutralization test (VNT), pseudo-virus neutralization test (pVNT). These methods are time consuming (need of several days), low through put and particularly require live biological materials or strict biosafety containment for testing. The recently developed methods such as recombinant VSV test significantly improved the assay in throughput. However, these methods are either still time-consuming or not quantitative.

To address this issue, EpigenTek has developed and offers the SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit. The kit has the following advantages and features:

- **Fast:** Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 50 minutes.
- **Robust:** Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent.
- **Sensitive and Specific:** The neutralizing antibody level can be detected from as low as 1 ng and is specific for SARS-CoV-2, which is suitable for identifying whether neutralizing antibody is present in infected individuals and distinguishing SARS-CoV-2 neutralizing antibody from other antibodies.
- **Quantitative:** The assay standard is included, which allows the neutralizing antibody titer or concentration to be quantified for EC_{50} calculation.
- **Flexible**: Strip-well microplate format makes the assay flexible for manual or high throughput analysis

References:

1. *Bunyavanich S et al: JAMA. Published online May 20, 2020.*
2. *Sama IZ et al: Eur Heart I, 41: 1810–1817, 2020.*
3. *Wang C et al: Natural Comm. 11: 2251, 2020*

PRINCIPLE & PROCEDURE

The SARS-CoV-2 spike protein contains ACE2 binding domain and is a major target of the host immune response. Virus entry and following replication and infection are dependent on interaction of spike protein with ACE2 receptor. Inhibition of spike protein function by neutralizing antibody will block the binding of spike protein to ACE2 and virus entry. Thus, detection of spike protein inhibition by neutralizing antibody would mimic the virus neutralization process and directly reflect the SARS-CoV-2- neutralizing antibody level.

The SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit contains all reagents necessary for quantitatively measuring SARS-CoV-2 neutralizing antibody level. In this assay, a SARS-CoV-2 spike protein is stably pre-coated onto microplate wells. His-tagged ACE2 is bound to the coated spike protein in the presence or absence of neutralizing antibody contained in the sample. The amount of the bound ACE2, which is proportional to ACE2 inhibition intensity, is then recognized by the Neutralizing Detection Complex containing anti-His moiety and measured through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The neutralizing antibody level is inversely proportional to the optical density intensity measured. The higher the neutralizing antibody is, the lower the OD intensity is.

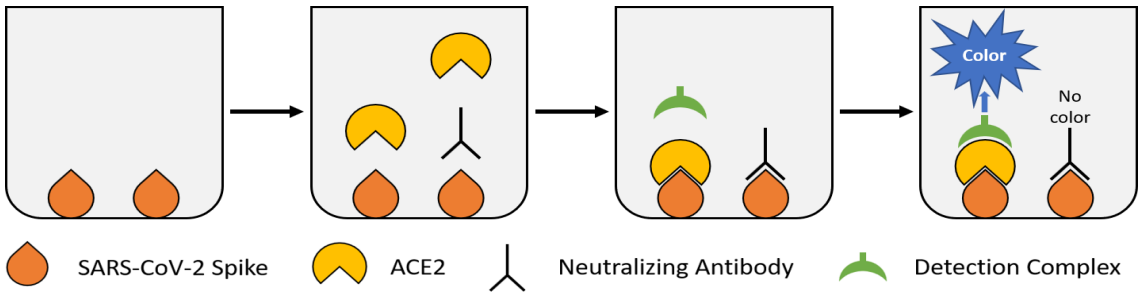

Fig 2. Schematic procedure of the SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit

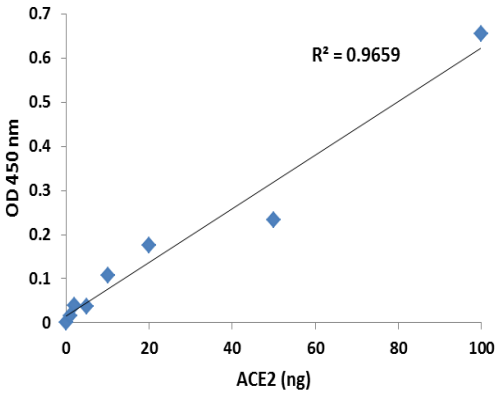

Fig 3. An example of an optimal standard curve generated with ACE2 protein and assayed with SeroFlash ™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit.

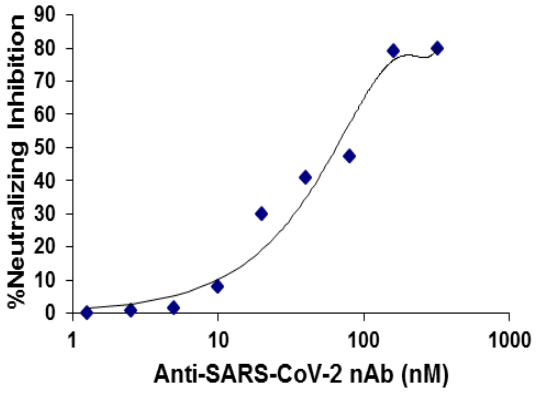

Fig 4. Inhibition of SARS-CoV-2-ACE2 binding by positive neutralizing antibody detected with the SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit.

ASSAY PROTOCOL

1. Buffer Solution & Preparation

a. Prepare **Diluted WB (**1X Wash Buffer):

48-Assay Kit: Add 13 ml of **WB** (10X Wash Buffer) to 117 ml of distilled water and adjust pH to 7.2-7.5.

96-Assay Kit: Add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 7.2-7.5.

This **Diluted WB** (1X Wash Buffer) can now be stored at 4°C for up to six months.

b. Prepare **Diluted NDC** (Neutralizing Detection Complex) solution:

Dilute **NDC** (Neutralizing Detection Complex) with **Diluted WB** (1X Wash Buffer) at 1:1000 ratio (ex: 1 µl of **NDC** + 1000 µl of **Diluted WB**).

c. Prepare **ACE2** standard:

Suggested Standard Curve Preparation: First, dilute **ACE2 (**His**-**ACE2 Protein) with **AB** (Assay Buffer) to 50 ng/µl by adding 2 µl of **ACE2** to 38 µl of **AB**. Then, further prepare seven concentrations by combining the 50 ng/µl **Diluted ACE2** with **AB** into final concentrations of 0.5, 1.0, 2.0, 5.0, 10, 25, and 50 ng/µl according to the following dilution chart:

Tube	Diluted ACE2 (50 ng/µl)	AB	Resulting Diluted ACE2 Concentration
1	1.0 µl	99.0 µl	0.5 ng/ µl
2	1.0 µl	49.0 µl	1.0 ng/µl
3	1.0 µl	24.0 µl	2.0 ng/µl
4	1.0 µl	9.0 µl	5.0 ng/µl
5	1.0 µl	4.0 µl	10 ng/µl
6	2.0 µl	2.0 µl	25 ng/µl
7	4.0 µl	0.0 µl	50 ng/µl

***Note**: (1) Keep each of diluted solutions except **Diluted WB** (1X Wash Buffer) on ice until use. Any remaining diluted solutions other than **Diluted WB** should be discarded if not used within the same day.*

2. Binding Inhibition Reaction

a. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (including blank and positive control) to ensure that the signal generated is validated. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

b. Blank Wells: Add 50 µl of **AB** (Assay Buffer) per well.

c. Positive Control Wells: Add 48 µl of **AB** (Assay Buffer) and 2 µl of **PC** (Positive Control)

d. Negative Control Wells: Add 48 µl of **AB** (Assay Buffer) and 2 µl of **NC** (Negative Control).

e. Standard Wells: Add 48 µl of **AB** (Assay Buffer) and 2 µl of **Diluted ACE2** standard at concentrations of 0.5 to 50 ng/µl (see the chart in Step 1c). The final concentrations should be 1, 2, 5, 10, 20, 50 and 100 ng per well.

f. No-Sample Control (NSC) Wells: Add 50 µl of **AB** (Assay Buffer) per well.

g. Sample Wells: Add 45 µl of **AB** (Assay Buffer), and 5 µl of undiluted sample solution (for qualitative assay) or diluted sample solution at different ratio (ex: 1:10, 1: 20, 1: 40…. 1: 1280, etc. for quantitative assay) with **AB** (Assay Buffer) per well.

h. Tightly cover the strip-well microplate with Parafilm M or aluminum foil to avoid evaporation, and incubate at 37°C for 20 min.

Note: *Generation of standard curve is optional. It may not be required if it is only for qualitative detection of neutralizing antibody contained in the samples such as screening if there is neutralizing antibody in serum.*

i. Meanwhile, Prepare **Working ACE2** (His-ACE2 Protein) solution: Dilute **ACE2** (His-ACE2 Protein) with **AB** (Assay Buffer) at 1:500 ratio (ex: 4 µl of **ACE2** + 2000 µl of **AB**). The final concentration of **Working ACE2** (His-ACE2 Protein) should be 2.0 µg/ml. Then add **NDC** (Neutralizing Detection Complex) into the diluted ACE2 at 1: 2000 ratio (ex: 1 µl of **NDC** +2000 µl of the diluted **ACE2**). Calculate the volume required for the assay. 50 µl of **Working ACE2** solution is required for each well.

j. After 20 min incubation, add 50 µl of the **Working ACE2** (His-ACE2 Protein) solution to each well except blank wells and standard wells. Add 50 µl of **Diluted NDC** (Neutralizing Detection Complex) to blank wells and standard wells. Now each well should contain 100 µl of solution

k. Tightly cover the strip-well microplate again with Parafilm M or aluminum foil to avoid evaporation, and incubate at 37°C for 25 min.

l. Remove the reaction solution from each well. Wash each well with 150 µl of **Diluted WB** (1X Wash Buffer) for a total of five times . This can be done by simply pipetting **Diluted WB** in and out of the wells.

3. Binding Inhibition Detection

a. Add 100 µl of **DS** (Developer Solution) to each well and incubate at room temperature for 2 min away from direct light. Monitor color change in the sample wells and control wells. The **DS** solution will turn blue in the presence of sufficient ACE2 protein.

b. Add 100 µl of **SS** (Stop Solution) to each well to stop the reaction when the color in the No-Sample-Control wells turns medium blue. Mix the solution by gently shaking the frame and wait 1 min to allow the color reaction to be completely stopped. The color will change to yellow after adding **SS** and absorbance should be read on a microplate reader within 2 to 10 min at 450 nm with an optional reference wavelength of 655 nm.

Note: *(1) Most microplate readers have the capability to carry out dual wavelength analysis and will automatically subtract the reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice – once at 450 nm and once*

at 655 nm. Then manualy subtract the 655 nm ODs from 450 nm ODs; (2) If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

4. Neutralizing Inhibition Calculation

a. Calculate the average duplicate readings for sample wells and blank wells.

b. Qualitative assay: simply calculate neutralizing inhibition% using the following formula:

$$\textit{Neutralizing Inhibition \%} = 1 - \frac{\textit{Sample OD} - \textit{Blank OD}}{\textit{NSC OD} - \textit{Blank OD}} \times 100\%$$

c. Quantitative assay: first, generate a standard curve and plot the OD values versus the amount of **ACE2** (His-ACE2 Protein) at each concentration point. Then determine the slope as OD/ng using linear regression (*Microsoft Excel's* linear regression or slope functions are suitable for such calculation) and the most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation. Now calculate neutralizing inhibition using the following formula:

$$\textit{Neutralizing Inhibition (ng/µl)*} = \frac{\textit{OD (NSC-blank)} - \textit{OD (sample – blank)}}{\textit{Slope} \times \textit{Sample volume (µl)}}$$

**As the molecular weight of ACE2 and the coated spike protein is similar and molar ratio of ACE2-spike protein binding is 1:1, thus the reduced binding amount of ACE2 to spike protein would be directly proportional to neutralizing inhibition intensity.*

When the standard curve is flat due to a saturated signal intensity at high concentration:

1) Graph the second order polynomial curve and obtain second order polynomial regression equation:

$$Y = aX^2 + bX$$

Here, X = neutralizing inhibition; Y = <Sample OD> – <Blank OD>; a and b is known Slope 1 and Slope 2, respectively.

** Microsoft Excel's polynomial regression function could be used for easy and convenient calculation.*

2) Calculate binding activity of the samples based on the following equation, derived from the above equation.

$$\text{Neutralizing Inhibition (ng/µl)} = \frac{(b^2 + 4aY)^{0.5} - b}{-2a} \div S$$

Here, **S** is the volume of input sample in µl.

d. Calculating IC50 (EC50): generate dose-response by plotting the OD values versus the sample (serum or antibody) at series concentration or titer dilution point. The IC50 or ED50 can be calculated with linear regression or four-parameter logistic regression based on the curve pattern.

5. Result interpretation

Quality control: for valid assay, the negative control delta ODs should be = NSC delta OD +/-10%; the positive control delta ODs should be < 80% of NSC delta ODs

Qualitative Assay: Neutralizing inhibition ≥ 20% indicates Positive and SARS-CoV-2 neutralizing antibody detected; ＜ 20% indicates Negative and No detectable SARS-CoV-2 neutralizing antibody.

Quantitative Assay: > 4 ng/µl indicates Positive, and SARS-CoV-2 neutralizing antibody detected. ＜ 4 ng/ µl indicates Negative, and no detectable SARS-CoV-2 neutralizing antibody.

IC50/ED50: 50% of neutralizing inhibition with antibody titer or concentration at the highest dilution of serum/plasma or purified antibody.

SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 1. Approximate amounts of required buffers and solutions for defined assay wells, based on the protocol.

Reagents	16 wells (2 strips)	48 wells (6 strips)	96 wells (12 strips)
Diluted WB (1X Wash Buffer)	25 ml	75 ml	150 ml
AB (Assay Buffer)	2 ml	6 ml	12 ml
ACE2 (His-ACE2 Protein)	2 µl	4 µl	8 µl
Diluted NDC (Neutralizing Detection Complex)	1 ml	3 ml	6 ml
DS (Developer Solution)	1.6 ml	5 ml	10 ml
SS (Stop Solution)	1.6 ml	5 ml	10 ml

SUGGESTED STRIPWELL SETUP

Table 2. The suggested strip-well plate setup for the binding activity assay in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicates.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	NC	NC	Sample	Sample
B	ACE2 1 ng	ACE2 1 ng	PC	PC	Sample	Sample
C	ACE2 2 ng	ACE2 2 ng	NSC	NSC	Sample	Sample
D	ACE2 5 ng	ACE2 5 ng	Sample	Sample	Sample	Sample
E	ACE2 10 ng	ACE2 10 ng	Sample	Sample	Sample	Sample
F	ACE2 25 ng	ACE2 25 ng	Sample	Sample	Sample	Sample
G	ACE2 50 ng	ACE2 50 ng	Sample	Sample	Sample	Sample
H	ACE2 50 ng	ACE2 50 ng	Sample	Sample	Sample	Sample

Note: *For qualitative assay, the* ***ACE2*** *(His-ACE2 Protein) standard curve is not required and all wells of strip 1 and 2 can be used for samples except blank wells.*

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in untreated sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Incorrect absorbance reading.	Check if appropriate absorbance wavelength (450 nm filter) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and the cap is tightly closed after each opening or use.
High background present in the blank wells	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by sample or positive control.	Ensure the wells are not contaminated from adding sample or positive control accidentally or from using contaminated tips.
	Over development of color.	Decrease the development time in Step 3a before adding **SS** (Stop Solution) in Step 3b.
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time.	Ensure **DS** (Developer Solution) and **SS (**Stop Solution) are added at the same time between replicates or otherwise maintain a consistent timing in between each addition of solutions.
	Color reaction is not evenly stopped due to an inconsistent order of adding solutions.	Ensure all solutions, particularly **DS** (Developer Solution) and **SS** (Stop Solution), are added in the same order each time as all other solutions.
	The solutions are not evenly added due to inconsistency in pipetting volume.	Ensure the solution in each pipette tip is equal in the multi-channel pipette. Equilibrate the pipette tip in any solutions before adding them. Ensure the solutions, especially those with small volumes (e.g., 1 ul) are completely added into the wells.

RELATED PRODUCTS

SARS-CoV-2 Serological Detection

D-1001 Seroflash™ SARS-CoV-2 IgM/IgG Antibody Detection Kit
D-1002 Seroflash™ SARS-CoV-2 IgM/IgG ELISA Fast Kit

SARS-CoV-2 Spike-ACE2 Binding Assay

D-1004	CoviDrop™ SARS-CoV-2 Spike-ACE2 Binding Inhibitor Screening Fast Kit
D-1005	CoviDrop™ SARS-CoV-2 Spike-ACE2 Binding Activity/Inhibition Assay Kit
D-1006	CoviDrop™ SARS-CoV-2 Targeted Preprotein Convertase Inhibitor Screening Fast Kit
D-1007	CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit