CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit

Base Catalog # D-1007

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit is designed for measuring SARS-CoV-2-targeted proprotein convertase (PC) cleavage activity and inhibition using various human samples so that the functional PC activity targeting SARS-CoV-2 S1/S2 site cleavage can be identified. The samples can be used immediately or stored at proper conditions for future use.

Input Material: Input materials can be cell/tissue extracts, serum, plasma, swab samples, and various body fluids.

Internal Control: A serine protease as the positive cleavage enzyme control is provided in this kit to determine if the kit works properly. Because activity and inhibition of SARS-CoV-2 targeted PCs can vary with various different inhibitors, 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-1007-48	96 Assays Cat. #D-1007-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
PAB (PC Assay Buffer)	6 ml	12 ml	RT
PCE (Positive Control Enzyme, 100 µg/ml)*	5 µl	10 µl	–20°C
CDS (Cleavage Detection Solution, 5000 X)*	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)	5	10	4°C
8-Well Blank Control Strips (Green Trimmed)	1	2	4°C
User Guide	1	1	RT

**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 **PCE** and **CDS** at –20°C away from light; (2) Store **WB**, **DS** and **8-well assay strips** at 4°C away from light; (3) Store all remaining components (**PAB** 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; and (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

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- ☐ Inhibitor of interest (Optional)
- ☐ 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. Thus, only use the User Guide that was supplied with the kit when using that kit.

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

Intellectual Property: This product and its 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. It was found that the SARS-CoV-2 spike glycoprotein harbors a furin/PC cleavage site at the boundary between the S_1/S_2 subunits, which could be cleaved by furin and/or furin-like PCs secreted from host cells and bacteria in the airway epithelium [1, 2]. Unlike SARS-CoV, cell entry of SARS-CoV-2 is pre-activated by furin and/or furin-like PCs, reducing its dependence on target cell proteases for entry [3]. The cleavage activation of S-protein is well demonstrated to be essential for SARS-CoV-2 spike-mediated viral binding to ACE2, cell-cell fusion, and viral entry into human lung cells [4, 5]. It was also observed that other viruses containing a furin/PC cleavage site, such as H5N1, increased replicates and developed higher pathogenicity [6].

The SARS-CoV-2 furin/PC cleavage site has been with one core region **SPRRAR**│**SV** (eight amino acids, P6–P2′). The core region is very unique, as its P2 or P3 position is a positively charged residue (Arg), and another residue is hydrophobic (Ala). These factors allow this site to be cleaved by furin or furin-like PC and the cleavage efficiency to be facilitated by other serine proteases targeting dibasic amino acid sites such as matriptase, plasmin, human airway trypsin (HAT), and TMPRSS2. Furthermore, a serine at P6 could also highly increase the cleavage efficiency, causing increased viral replication, unrestricted organ tropism, virulence, and a rise in the mortality rate as proven in H5N1 infection in mice [7].

The importance of measuring SARS-CoV-2S1/S2 site targeted cleavage PC or facilitating protease activity is emphasized by that the PC-based SARS-CoV-2 S1/S2 cleavage increases SARS-Cov-2 entry into cells and replication and eventually develops higher pathogenicity of COVID-19.

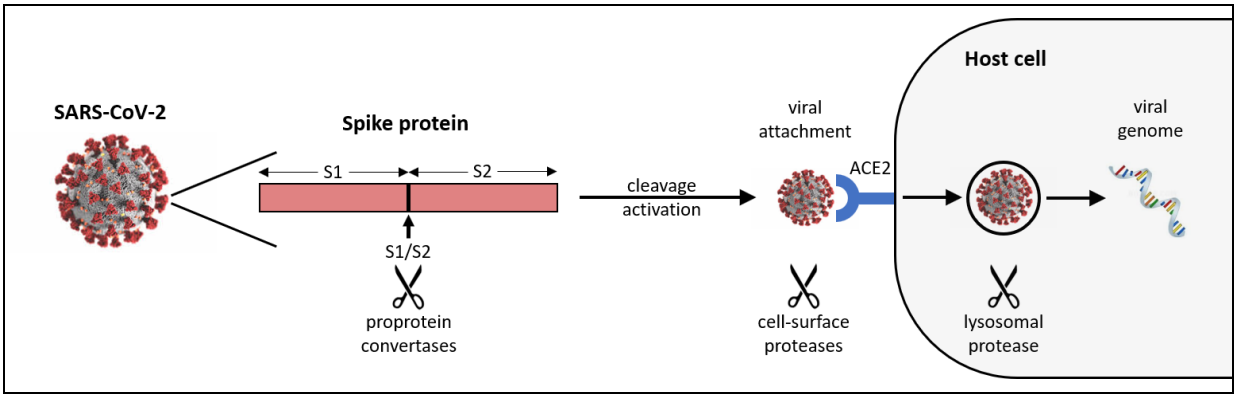

Fig 1. Sars-Cov-2 spike protein cleavage by proprotein convertases and other proteases during entry into cells.

There are no/few methods available for measuring SARS-CoV-2 targeted PC or related protease cleavage activity and inhibition. To address this issue, EpiGentek developed the CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay 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 min.
- **Robust:** Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent.
- **Sensitive:** The activity can be detected from as low as 2 ng of PC (ex: furin) or facilitated serine protease.
- **Specific:** The substrate contains entire SARS-CoV-2-S1/S2 cleavage sequence and is proven to be the same as complete trimeric form of full-length SARS-CoV-2 spike protein in PC cleavage tests. Thus, the assay is uniquely specific for detecting SARS-CoV-2-S1/S2 cleavage and is suitable for measuring furin or furin-like PC as well as facilitated protease cleavage activity /inhibition using various biological samples.
- **Convenient:** Both purified proteins and biological samples, including cell/tissue extracts, plasma, serum, other body fluids, and, particularly, swab samples can be used, which allows the detection of PC and facilitating protease activity and inhibition.
- **Flexible:** Strip-well microplate format makes the assay flexible for manual or high throughput analysis.

Reference:

1. *Andersen KG et al: Nat Med. 26: 450-452, 2020.*
2. *Coutard B. et al: Antiviral Research, 176, 2020.*
3. *Shang J et al: Proc Natl Acad Sci. 117: 11727-11734, 2020*
4. *Hoffmann M et al: Mol Cell, 78:779-784, 2020.*
5. *Hoffmann M et al: Cell. 181:271-280, 2020.*
6. *Decha P. et al: Biophys J. 95: 128–134, 2008.*
7. *Zhang Y et al: J Virol. 86: 6924–6931, 2012.*

PRINCIPLE & PROCEDURE

The SARS-CoV-2 Targeted Proprotein Convertase Activity and Inhibition Assay Kit contains all reagents necessary for the measurement of SARS-CoV-2-targeted PCs and facilitating proteases activity and/or inhibition. In this assay, a SARS-CoV-2 specific substrate is tagged with polyhistidine at N-terminal and biotin at C-terminal and bound onto microplate wells through histidine/Ni-NTA at its N-terminal. The cleavage of the substrate at S1/S2 site will remove the S2 part (C-terminal) of the substrate after washing, which causes a decrease in the signal generated by avidin/biotin binding after adding Cleavage Detection Solution (CDS). The inhibition of the cleavage by inhibitors will block the reduction of the signals. The signal intensity is measured through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The more the substrate is cleaved, the lower the signal generated will be. Thus, the PC cleavage activity is inversely proportional to the signal intensity.

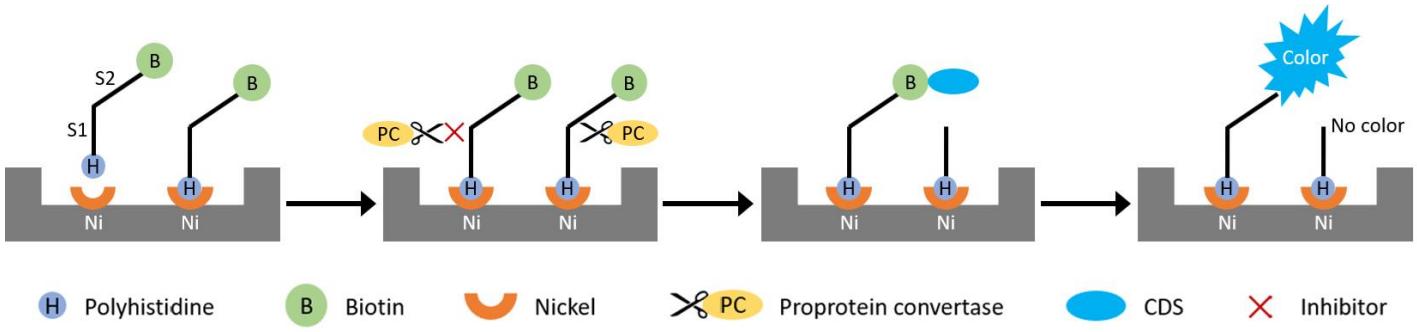

Fig 2. Schematic procedure of the CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit.

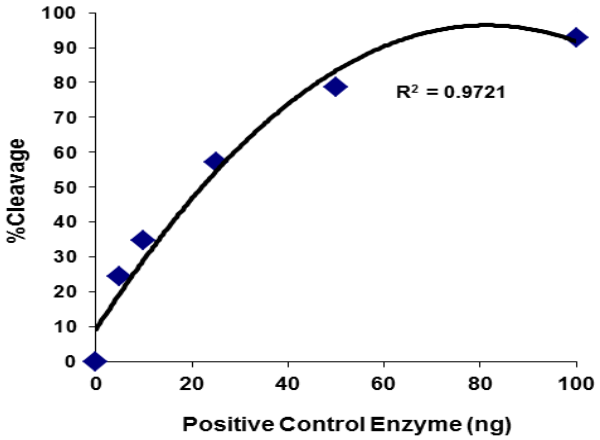

Fig 3. Illustrated dose-response of Positive Control Enzyme cleavage activity assayed with the CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit Activity/Inhibition Assay Kit.

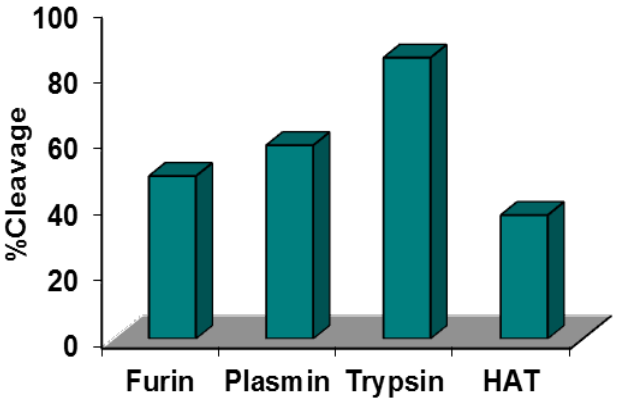

Fig 4. SARS-CoV-2-targeted protease cleavage activity detected using the CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit Concentration (ng/well): Furin: 10 ng; Plasmin: 50 ng; Trypsin: 50 ng. HAT: 200 ng.

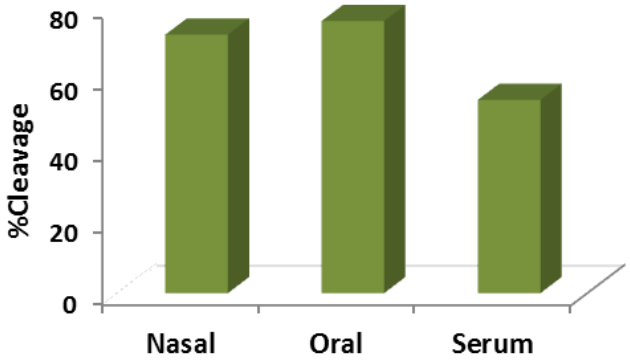

Fig 4. SARS-CoV-2-targeted protease cleavage activity and inhibition detected by the CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit with use of human biological samples. Nasal and Oral swab sample: released into 300 µl of **PAB** and 30 µl of sample solution was used for assay. Human serum sample: 5 µl.

ASSAY PROTOCOL

Starting Materials

Input Amount: For whole cell/tissue extract, plasma, serum, or body fluid, the sample volume should not be more than 5 µl (10% of total assay solution). For swab samples, the sample can be collected according to the standard procedure of swab sample collection and released into 300 µl of **PAB** (PC Assay Buffer, #D1007-PAB, EpiGentek) by rotating the swab in **PAB** (PC Assay Buffer) for 30 seconds and then used for the assay with 30 µl of the sample solution. For the purified PC or facilitating protease proteins, the amount can be 0.5 ng to 200 ng, depending on the purity and properties of the protein (wild type or mutated one).

Whole Cell Extraction: You can use your own method of choice for preparing whole cell extracts. EpiGentek also offers a whole cell extraction kit (Cat. No. OP-0003) optimized for use with this kit.

Cell Extract or Purified protein Storage: Cell extract should be stored at –80°C. The purified proteases should be stored at -80°C or according to the supplier's instruction until use.

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 CDS** (Cleavage Detection Solution):

Dilute **CDS** (Cleavage Detection Solution) with **Diluted WB** (1X Wash Buffer) at a dilution of 1:5000 (i.e., add 1 µl of **CDS** to 5000 µl of **Diluted WB**). About 50 µl of this **Diluted CDS** will be required for each assay well.

***Note**: 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. Cleavage and Inhibition Reaction

a. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include 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 (Green Trimmed): Add 50 µl of **PAB** (PC Assay Buffer) per well.

c. No-Enzyme Control (NEC) Wells: Add 50 µl of **PAB** (PC Assay Buffer) per well

d. Positive Control Enzyme Wells: Add 49 µl of **PAB** (PC Assay Buffer) and 1 µl of **PCE** (Positive Control Enzyme) per well.

e. Sample Wells Without Inhibitor: For whole cell extraction, plasma/serum and body fluid, Add 45 µl of **PAB** (PC Assay Buffer) per well followed by adding 5 µl of the sample; For swab sample released into **PAB** (PC Assay Buffer), add 20 µl of **PAB** (PC Assay Buffer) per well followed by adding 30 µl of sample solution.

f. Sample Wells With Inhibitor: For whole cell extraction, plasma/serum, and body fluid, add 40 µl of **PAB** (PC Assay Buffer), 5 µl of the sample, and 5 µl of inhibitor solution per well. For swab sample released into **PAB** (PC Assay Buffer), add 15 µl of **PAB** (PC Assay Buffer) per well followed by adding 30 µl of sample solution and 5 µl of inhibitor solution per well.

g. Tightly cover the strip-well microplate with Parafilm M or aluminium foil to avoid evaporation, and incubate at room temperature for 25 min.

***Note:** (1) Follow suggested well setup diagrams; (2) The concentration of inhibitors to be added into the sample wells can be varied. However, the final concentration of the inhibitors before adding to the wells should be prepared with **PAB** (PC Assay Buffer), at a 1:9 ratio (e.g., add 0.5 µl of inhibitor to 4.5 µl of **PAB**), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

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

3. Cleavage Activity and Inhibition Detection

a. Add 50 µl of the **Diluted CDS** (Cleavage Detection Solution) to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 15 min.

b. Remove the **Diluted CDS** (Cleavage Detection Solution) from each well.

c. Wash each well with 150 µl of the **Diluted WB** (1X Wash Buffer) for a total of five washes.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed as much as possible at each wash step.*

d. 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 un-cleaved substrate.

e. Add 100 µl of **SS** (Stop Solution) to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. Mix the solution by gently shaking the frame and wait 2 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. Cleavage Inhibition Calculation

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

b. Simply calculate cleavage% using the following formula:

$$\textit{Cleavage\%} = \left[1 - \frac{\textit{[OD (NEC – blank) – OD (sample – blank)]}}{\textit{OD (NEC – blank)}}\right] \times 100\%$$

c. Calculate cleavage activity of PCs or proteases using the following formula (suitable for whole cell extracts and purified proteases):

$$\textit{Cleavage activity (OD/min/µg)} = \frac{\textit{[OD (NEC – blank) – OD (sample – blank)]}}{\textit{OD (NEC-Blank) X [Protein Amount (ng)/1000]* X min **}}$$

* Protein amount added into the reaction at step 2e.
** Incubation time at step 2g (in minutes).

d. Calculate cleavage activity inhibition using the following formula:

$$\textit{PC Inhibition \%} = \frac{\textit{[OD (control – blank) – OD (inhibitor sample – blank)]}}{\textit{[OD (control – blank) – OD (no inhibitor sample – blank)]}} \times 100\%$$

SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 1. Approximate amount 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
PAB (PC Assay Buffer)	2 ml	6 ml	12 ml
PC (Positive Control Enzyme)	1 µl	2 µl	2-4 µl
CDS (Cleavage Detection Solution)	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	Sample	Sample	Sample	Sample
B	PCE	PCE	Sample	Sample	Sample	Sample
C	NEC	NEC	Sample	Sample	Sample	Sample
D	Sample	Sample	Sample	Sample	Sample	Sample
E	Sample	Sample	Sample	Sample	Sample	Sample
F	Sample	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in No-Enzyme Control 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 capped after each opening or use.
High background present in the blank wells	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.
	Over development of color.	Decrease the development time in Step 3d before adding **SS** (Stop Solution) in Step 3e.
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 using 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
D-1008 SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay 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

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