CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening Kit

Base Catalog # D-1010

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening Kit is designed for screening Blockers of SARS-CoV-2 specific furin cleavage site (FCS) and/or inhibitors of furin and other serine proteases such as human air trypsin (HAT) that may also target the FCS of SARS-CoV-2, in a fast and high throughput format.

Input Material: Input materials can be small molecule compounds and other biological molecules that block SARS-CoV-2 specific FCS and/or inhibit furin and serine protease activity against SARS-CoV-2 specific FCS.

Internal Control: A serine protease as the cleavage enzyme control is provided in this kit to determine if the kit works properly. Because blocking SARS-CoV-2 specific FCS or inhibiting furin activity can vary with various different blockers/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	96 Assays Cat. #D-1010-096	2x96 Assays Cat. #D-1010-192	Storage Upon Receipt
WB (10X Wash Buffer)	28 ml	2 x 28 ml	4°C
PAB (PC Assay Buffer)	12 ml	24 ml	RT
PCE (Positive Control Enzyme, 100 µg/ml)*	10 µl	20 µl	–20°C
CDS (Cleavage Detection Solution, 2000 X)*	8 µl	16 µl	–20°C
DS (Developer Solution)	10 ml	2 x 10 ml	4°C
SS (Stop Solution)	10 ml	2 x 10 ml	RT
8-Well Assay Strips (With Frame)	12	2 x 12	4°C
8-Well Blank Control Strips (Green Trimmed)	2	4	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 **PCE** and **CDS** at –20°C away from light; (2) Store **WB**, **DS, 8-well assay strips,** and **8-Well Blank Control 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
- ☐ PCs or proteases of interest

- ☐ Blocker/Inhibitor of interest
- ☐ Parafilm M or aluminum 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. It was found that the SARS-CoV-2 spike glycoprotein harbors a furin cleavage site (FCS) 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].

The SARS-CoV-2 furin/PC cleavage site has one core region **SPRRAR**│**SV** (eight amino acids, P6–P2′). The core region is very unique as its P2 and P3 positions are positively charged residues (Arg), and another residue is hydrophobic (Ala). This status allows such sites to be cleaved by furin or furin-like PC and the cleavage efficiency facilitated by other serine proteases targeting dibasic amino acid sites such as human airway trypsin (HAT).

It is well known that SARS-CoV-2 S1/S2 cleavage increases SARS-CoV-2 entry into cells and replication, eventually leading to higher transmission and pathogenicity of COVID-19. The mutation of P681 (non-polar proline) to positively charged R681 (arginine) to form multi-basic amino acid sites could further increase S1/S2 boundary cleavage, thereby increasing viral replicates in human airway and transmission [6]. Particularly, the P681R mutation is a hallmark of the Delta variant, the dominant strain of the COVID-19 global pandemic, and it leads to increased resistance of SARS-CoV-2 to wild-type spike vaccine [7]. Thus, inhibition of SARS-CoV-2

cleavage of FCS containing P681R would allow for the reduction of FCS cleavage-based activation of SARS-CoV-2 delta variant spike protein, thereby decreasing viral binding to ACE2, cell-cell fusion, and viral entry into human cells. There are two approaches to block or reduce cleavage of P681R mutated FCS of the spike proteins. One approach is based on the inhibition of furin or furin-like protease activity against FCS. For example, peptide or small molecular inhibitors could be used to inhibit the activity of furin or furin-like proteases. Another one is to block SARS-CoV-2 P681R FCS directly. For example, antibody or peptide ligands could be used as blockers against P681R FCS, avoiding or reducing the disadvantages of inhibiting host furin and furin-like proteases as such inhibition could non-specifically cause damage to the normal functions of the proteins that need activation by these enzymes.

Screening blockers and/or inhibitors of SARS-CoV-2 P681R mutated FCS could help develop effective drugs for COVID-19 therapy by blocking SARS-CoV-2 cleavage at S1/S2 boundary site.

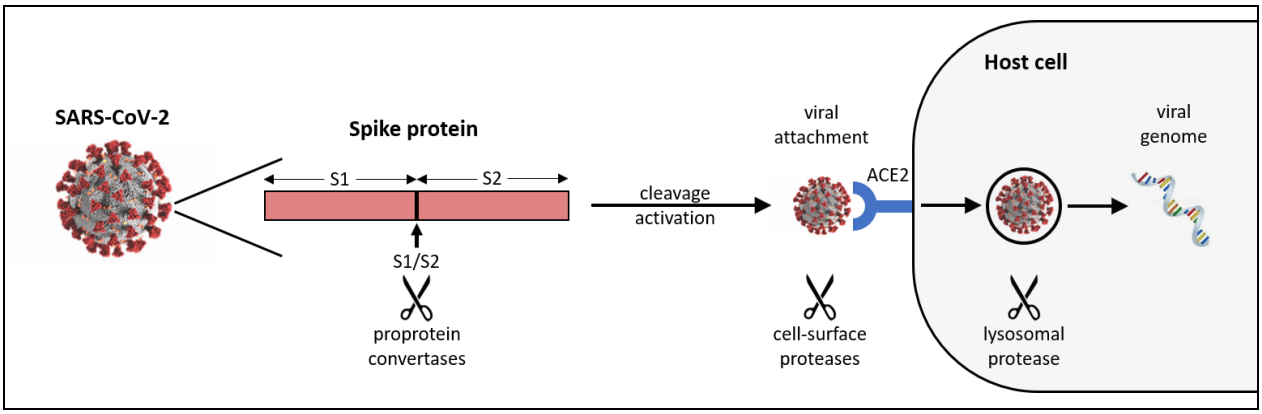

Fig 1. Sars-CoV-2 spike protein cleavage by furin and other proteases during entry into cells.

There are no/few methods available for screening both blockers of SARS-CoV-2 S1/S2 site cleavage and inhibitors of furin and furin-like proteases. To address this issue, EpigenTek developed and offers the CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening 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 1 hour and 10 min.
- **Robust:** Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent.
- **Dual-Function:** The kit allows for screening of both SARS-CoV-2 P681R mutated FCS direct blockers or furin and furin-like protease activity inhibitors.
- **Specific:** The substrate contains the entire SARS-CoV-2-S1/S2 cleavage sequence with P681R mutation. Thus, the assay is uniquely specific for detecting SARS-CoV-2 P681R mutated FCS cleavage and its inhibition by various blockers/inhibitors including small molecule chemicals, therapeutic peptides, antibodies, or other biological inhibitors.
- **Flexible:** Stripwell 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. Saito A et al: BioRxiv. Aug, 2021.
7. Planas D et al: Nature, 596: 276-280, 2021.

PRINCIPLE & PROCEDURE

The CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening Kit contains all reagents necessary for screening various blockers/inhibitors against SARS-CoV-2-FCS. 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 the decrease in 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 that will be generated. Thus, the PC cleavage activity is inversely proportional to the signal intensity.

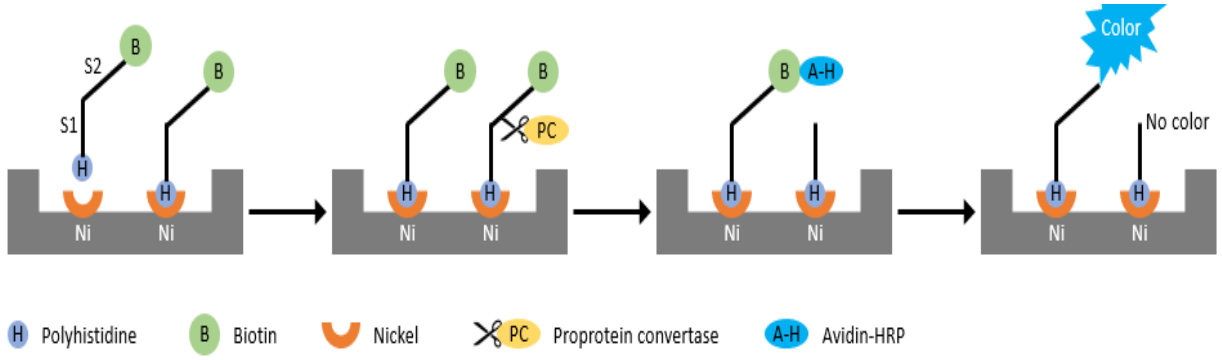

Fig 2. Schematic procedure of the CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening Kit.

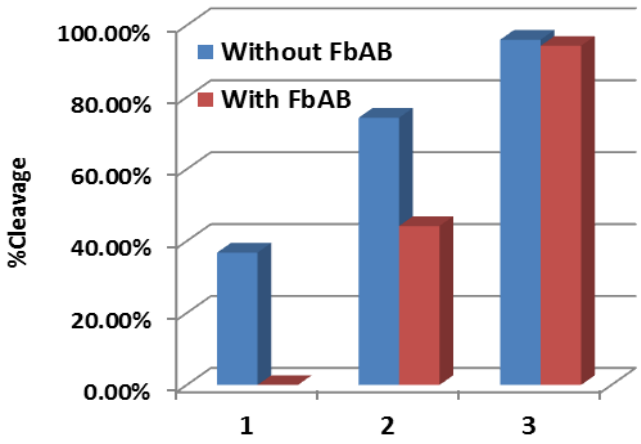

Fig 3. Blockage of protease cleavage of SARS-CoV-2 P681R mutated FCS by furin site blocking antibody (FbAB). 1. Furin: 8 ng/well; 2. Furin: 16 ng/well; and 3. Trypsin: 10 ng/well. fbAB: 200 ng/well.

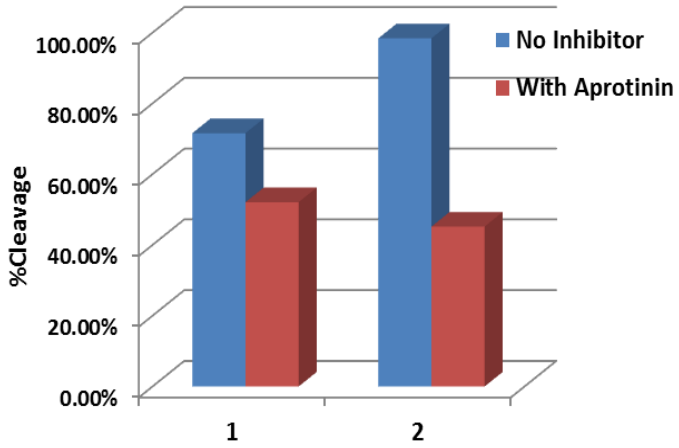

Fig4. SARS-CoV-2-targeted protease cleavage inhibition detected using the CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (P681R Mutation) Blocker/Inhibitor Screening Kit. 1. Furin: 16 ng/well; 2. Trypsin: 10 ng/well. Aprotinin: 500 ng/well.
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ASSAY PROTOCOL

1. Buffer Solution & Preparation

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

For 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.

For 2 X 96 Assay Kit: Add 52 ml of **WB (10X Wash Buffer)** to 468 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 ratio of 1:2000 (i.e., add 1 µl of **CDS** to 2000 µl of **Diluted WB**). About 50 µl of this **Diluted CDS** will be required for each assay well.

c. Prepare **Working PCE or Working Protease Solution**:

Working PCE: Add 1 µl of PCE to each 49 µl of **PAB** (PC assay buffer).

Working Protease Solution: Dilute protease of interest with **PAB (PC Assay Buffer)** to the desired concentration. 50 µl of working PCE or protease solution are required per sample well. Original solvent of the protease should be < 10% of the **Working Protease Solution**.
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.*

The anticipated approximate volumes of reagents needed are reflected below for this assay.

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)	96 wells (2 x 12 strips)
Diluted WB (1X Wash Buffer)	25 ml	75 ml	150 ml	350 ml
PAB (PC Assay Buffer)	1.5 ml	5 ml	10 ml	20 ml
PCE (Positive Control Enzyme)	1 µl	2 µl	2-4 µl	4-8 µl
Diluted CDS (Cleavage Detection Solution)	1 ml	3 ml	6 ml	12 ml
DS (Developer Solution)	1.6 ml	5 ml	10 ml	20 ml
SS (Stop Solution)	1.6 ml	5 ml	10 ml	20 ml

2. Suggested Stripwell Plate Setup

a. Review the configuration of the **suggested strip-well plate setup** in **table 2**.

Table 2. The suggested strip-well plate setup for the inhibitor screening 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

b. Predetermine the number of stripwells 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 unneeded stripwells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

3. Cleavage and Inhibition Reaction

I. For screening blockers against SARS-CoV-2 FCS:

a. Blank Wells **(Green Trimmed):** Add 50 µl of **PAB (PC Assay Buffer)** per well.

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

c. Positive Control Enzyme Wells: Add 50 µl of **PAB (PC Assay Buffer)** per well.

d. Sample Wells Without blocker: Add 50 µl of **PAB (PC Assay Buffer)** per well.

e. Sample Wells With blocker: Add 45 µl of **PAB (PC Assay Buffer)**, and 5 µl of blocker solution per well.

f. Tightly cover the strip-well microplate with Parafilm M or aluminum foil to avoid evaporation, and incubate at room temperature for 10 min.

Note: *(1) Follow suggested well setup diagrams; (2) The concentration of blocker to be added into the sample wells can be varied. However, the final concentration of the blocker before adding to the wells should be prepared with* ***PAB (PC Assay Buffer)****, at a 1:10 ratio (e.g., add 0.5 µl of blocker to 4.5 µl of* ***PAB****), so that the original solvent of the blocker can be reduced to 1% of the reaction solution or less.*

g. After 10 min incubation, add 50 µl of the **Working PCE** to positive control enzyme wells, 50 µl of **Working Protease Solution** to each sample well. Add 50 µl of **PAB (PC Assay Buffer)** to blank wells and no-enzyme control wells.

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

i. Remove the reaction solution from each well. Wash each well two times with 150 µl of **Diluted WB (1X Wash Buffer)** each time.

II. For screening inhibitors of furin or furin-like proteases:

Add 50 µl of Working Protease Solution in a 0.2 ml PCR tube followed by adding 5.5 µl of inhibitors and incubate at 37°C for 10 min. After 10 min incubation:

a. Blank Wells (Green Trimmed): Add 50 µl of **PAB (PC Assay Buffer)** per well

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

c. Positive Control Enzyme Wells: Add 50 µl of the **Working PCE** per well

d. Sample Wells Without Inhibitor: Add 50 µl of **Working Protease Solution** per well

e. Sample Wells With Inhibitor: Add 50 µl of **Working Protease Solution** incubated with inhibitors at step3 II a per well

Note: *(1) Follow suggested well setup diagrams; (2) The concentration of inhibitors to be added into the* ***Working Protease Solution*** *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 1:10 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.*

f. Tightly cover the stripwell microplate with Parafilm M or aluminum foil to avoid evaporation, and incubate at 37°C for 25 min.

g. Remove the reaction solution from each well. Wash each well two times with 150 µl of **Diluted WB (1X Wash Buffer)** each time.

4. Cleavage Inhibition Detection

a. Add 50 µl of the **Diluted CDS (Cleavage Detection Solution)** to each well, then carefully cover with Parafilm M or aluminum foil and incubate at room temperature for 20 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)** each time for five times.

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 1-4 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.*

5. Cleavage Inhibition Calculation

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

b. Calculate cleavage activity inhibition using the following formula:

$$\textit{FCS Inhibition \%} = \frac{\textbf{[OD (NEC – blank) – OD (blocker/inhibitor sample – blank)]}}{\textbf{[OD (NEC – blank) – OD (no blocker/inhibitor sample – blank)]}} \textbf{ x } \textit{100\%}$$

Note: *OD for blocker/inhibitor sample may be > OD for NEC and it should be considered as 100% inhibition under this condition.*

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in NEC 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 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 well is not contaminated from adding sample or positive control accidentally or from using contaminated tips.
	Over development of color.	Decrease the development time in Step 4d before adding **SS (Stop Solution)** in Step 4e.
Large variation between replicate wells	Color reaction not evenly stopped due to inconsistent 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 not evenly stopped due to 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 IgG/IgM 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 Proprotein Convertase Inhibitor Screening Fast Kit
D-1007 CoviDrop™ SARS-CoV-2 Targeted Proprotein Convertase Activity/Inhibition Assay Kit
D-1008 SeroFlash™ SARS-CoV-2 Neutralizing Antibody Assay Fast Kit
D-1009 CoviDrop™ SARS-CoV-2 Specific Furin Cleavage Site (Wild type) Blocker/Inhibitor Screening Kit