SARS-CoV-2 Main Protease Inhibitor Screening Assay Kit

Item No. 701960

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/ Size	Storage
701961	SARS-CoV-2 Main Protease Assay Buffer	1 vial/25 ml	-20°C
701963	SARS-CoV-2 Main Protease Enzyme (recombinant)	1 vial/40 µl	-80°C
701964	SARS-CoV-2 Main Protease Inhibitor (GC376)	1 vial/100 µl	-20°C
701962	SARS-CoV-2 Main Protease Substrate	1 vial/250 µl	-20°C
700416	DTT (1 M) Assay Reagent	1 vial/1 ml	-20°C
400091	Half-Volume 96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence with excitation and emission wavelengths of 340 and 490 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of ultrapure water, with a resistivity of 18.2 MΩ-cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Microcentrifuge tubes

INTRODUCTION

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped positive-stranded RNA virus and the causative agent of COVID-19, a primarily respiratory illness characterized by fever, cough, and shortness of breath that can lead to life-threatening complications.[1-5] The SARS-CoV-2 genome contains approximately 30 kilobases and 14 open reading frames (ORFs) that encode four structural proteins: spike, envelope, membrane, and nucleocapsid, as well as 16 non-structural proteins and 9 accessory factors.[6] The SARS-CoV-2 main protease (M^{pro}), also known as 3C-like protease ($3CL^{pro}$), is encoded within the non-structural protein 5 (nsp5) region of *ORF1ab*.[6] Auto-activation of SARS-CoV-2 M^{pro} cleaves nsp5-16, which, together with nsp1-4, form the SARS-CoV-2 replication and transcription complex (RTC), which is critical for the viral replication cycle.[6] Inhibition of SARS-CoV-2 M^{pro} activity reduces SARS-CoV-2 replication in infected cells *in vitro*, as well as decreases lung viral loads and pulmonary lesions in *ACE2* transgenic mice infected with SARS-CoV-2, indicating the therapeutic potential of targeting M^{pro} in the management of SARS-CoV-2 infections.[7,8]

About This Assay

Cayman's SARS-CoV-2 Main Protease Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of SARS-CoV-2 M^{pro}, one of the proteases required for processing viral polyproteins into non-structural proteins. The assay uses a SARS-CoV-2 M^{pro}-specific fluorogenic substrate containing a cleavage site found between nsp4 and nsp5. SARS-CoV-2 M^{pro} cleaves this substrate separating an EDANS fluorophore from a Dabcyl quencher generating free EDANS, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 340 and 490 nm, respectively. The SARS-CoV-2 M^{pro} inhibitor GC376 is included as a positive control.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted SARS-CoV-2 Main Protease Assay Buffer at a concentration 10X the desired final assay concentration (*e.g.*, for 20 µM final assay concentration, a 200 µM stock should be made). This solution may contain up to 50% DMSO, dimethyl formamide (DMF), or short chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤5% (see 'Effects of Solvents' on page 17).

Reagent Preparation

1. **SARS-CoV-2 Main Protease Assay Buffer**

   Mix 12.5 ml of SARS-CoV-2 Main Protease Assay Buffer (Item No. 701961) with 50 µl of the supplied DTT (1 M) Assay Reagent (Item No. 700416). The SARS-CoV-2 Main Protease Assay Buffer with DTT should be discarded if not used within the same day. Once thawed, the SARS-CoV-2 Main Protease Assay Buffer without DTT may be stored at 4°C for at least one month.

2. **SARS-CoV-2 Main Protease Substrate**

   Mix 200 µl SARS-CoV-2 Main Protease Substrate (Item No. 701962) with 1.8 ml SARS-CoV-2 Main Protease Assay Buffer. The diluted substrate will be stable at room temperature for two hours. If all of the SARS-CoV-2 Main Protease Substrate will not be used at one time, aliquot the undiluted substrate and store at -20°C protected from light where it will be stable for at least 1 month.

3. **SARS-CoV-2 Main Protease Enzyme (recombinant)**

   SARS-CoV-2 Main Protease Enzyme (Item No. 701963) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 25 µl of SARS-CoV-2 Main Protease Enzyme (recombinant) with 1.975 ml SARS-CoV-2 Main Protease Assay Buffer. It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme loses 10% of its activity when stored on ice for two hours. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

4. **SARS-CoV-2 Main Protease Inhibitor (GC376)**

   Mix 10 µl of SARS-CoV-2 Main Protease Inhibitor (GC376) (Item No. 701964) with 90 µl SARS-CoV-2 Main Protease Assay Buffer to make a 500 µM working solution. If all of the SARS-CoV-2 Main Protease Inhibitor (GC376) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

ASSAY PROTOCOL

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control SARS-CoV-2 Main Protease Inhibitor (GC376), be assayed in triplicate. It is suggested that the contents of each well be recorded on the template sheet provided on page 21. A typical layout of samples to be measured in triplicate is shown in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all the wells.
- Use the SARS-CoV-2 Main Protease Assay Buffer with DTT in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended to assay the samples in triplicate, but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 340 nm and an emission wavelength of 490 nm.

Performing the Assay

1. **Background Wells:** Add 70 µl SARS-CoV-2 Main Protease Assay Buffer and 10 µl of solvent (the same solvent concentration used to dissolve the unknown inhibitor and the positive control SARS-CoV-2 Main Protease Inhibitor (GC376)) to three wells. Mix the contents of the wells by pipetting.
2. **100% Initial Activity Wells:** Add 50 µl of SARS-CoV-2 Main Protease Assay Buffer, 20 µl of SARS-CoV-2 Main Protease Enzyme, and 10 µl of solvent to three wells. Use the same solvent concentration used for the unknown inhibitor and the positive control, SARS-CoV-2 Main Protease Inhibitor (GC376). Mix the contents of the wells by pipetting.
3. **Inhibitor/Positive Control Wells:** Add 50 µl of SARS-CoV-2 Main Protease Assay Buffer, 20 µl of SARS-CoV-2 Main Protease Enzyme, and 10 µl of unknown inhibitor or the 500 µM positive control, SARS-CoV-2 Main Protease Inhibitor (GC376), working solution to three wells. Mix the contents of the wells by pipetting.
4. Incubate for 30 minutes at room temperature.
5. Initiate the reactions by adding 20 µl of SARS-CoV-2 Main Protease Substrate to all the wells being used and mix well by pipetting.
6. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for two hours at room temperature protected from light.
7. Remove the plate cover and read the plate with an excitation wavelength of 340 nm and an emission wavelength of 490 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples.

ANALYSIS

Calculations

1. Determine the average fluorescence (AF) of each sample.
2. Subtract the AF of the background wells from the AF of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\%\text{Inhibition} = \left[\frac{(\text{corrected 100\% initial activity} - \text{corrected inhibitor activity})}{\text{corrected 100\% initial activty}}\right] \times 100$$

$$\%\text{Activity} = \left[\frac{(\text{corrected inhibitor activity})}{\text{corrected 100\% initial activty}}\right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of recombinant SARS-CoV-2 Main Protease by SARS-CoV-2 Main Protease Inhibitor (GC376) is shown in Figure 2 (see page 15).

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[9]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{| \mu_{c+} - \mu_{c-} |}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z´ factor is 1.0. A robust assay has a Z´ factor >0.5. The Z´ factor for Cayman's SARS-CoV-2 Main Protease Inhibitor Screening Assay Kit was determined to be 0.85.

Sample Data:

The data presented here is an example of data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

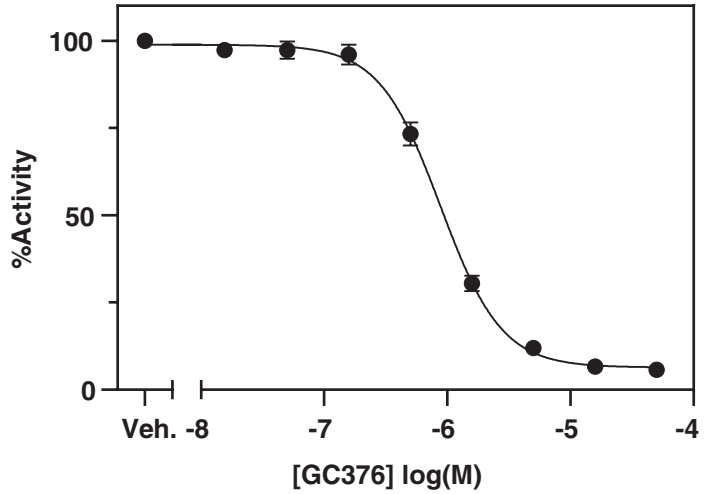

Figure 2. Inhibition of recombinant SARS-CoV-2 Main Protease by SARS-CoV-2 Main Protease Inhibitor (GC376). Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity.

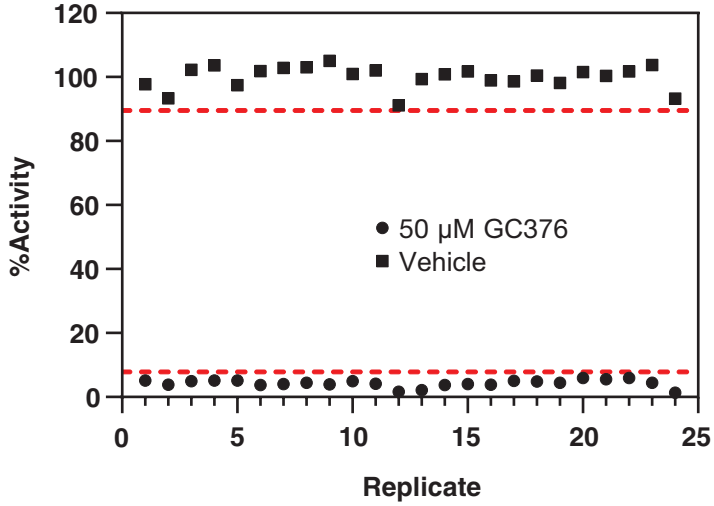

Figure 3. Typical Z′ data for the SARS-CoV-2 Main Protease Inhibitor Screening Assay Kit. Data are shown from 24 replicates each for vehicle control (Veh.) and 50 µM SARS-CoV-2 Main Protease Inhibitor (GC376) prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.85. The red lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (*e.g.* MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤5%. A titration of organic solvents showed that the signal changes with solvent concentration so the proper vehicle control should be included in the assay.

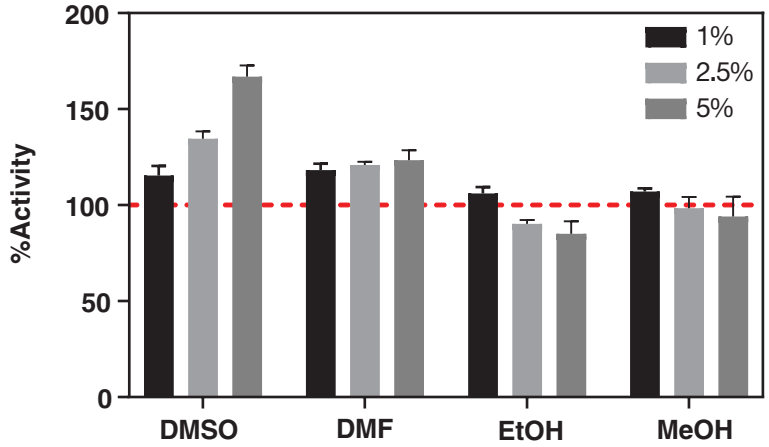

Figure 4. The effect of solvent on the readout of SARS-CoV-2 Main Protease activity. The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents. The red dotted line at 100% is the buffer control.

Precision:

Intra-assay precision was determined by analyzing 24 measurements of the background, vehicle, and 50 µM SARS-CoV-2 Main Protease Inhibitor (GC376) on the same day. The intra-assay coefficients of variation were 3, 2.4, and 2.4%, respectively. The intra-assay coefficient of variation for the IC_{50} value of five inhibition curves performed on the same day was 7.7%.

Inter-assay precision was determined by analyzing inhibition with SARS-CoV-2 Main Protease Inhibitor (GC376) in separate assays on four different days. The inter-assay coefficient of variance for the IC_{50} value was 4.7%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the inhibitor wells	A. Substrate was not added to the wells B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all the components to the well(s) and re-assay B. Reduce the inhibitor concentration and re-assay
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the compound concentration and re-assay

References

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2. Lu, R., Zhao, X., Li, J., *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* **395(10224)**, 565-574 (2020).
3. Meo, S.A., Alhowikan, A.M., Al-Khlaiwi, T., *et al.* Novel coronavirus 2019-nCoV: Prevalence, biological and clinical characteristics comparison with SARS-CoV and MERS-CoV. *Eur. Rev. Med. Pharmacol. Sci.* **24(4)**, 2012-2019 (2020).
4. Klok, F.A., Kruip, M.J.H.A., van der Meer, N.J.M., *et al.* Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb. Res.* **191**, 145-147 (2020).
5. Yang, F., Shi, S., Zhu, J., *et al.* Analysis of 92 deceased patients with COVID-19. *J. Med. Virol.* **92(11)**, 2511-2515 (2020).
6. Romano, M., Ruggiero, A., Squeglia, F., *et al.* A structural view of SARS-CoV-2 RNA replication machinery: RNA synthesis, proofreading and final capping. *Cells* **9(5)**, 1267 (2020).
7. Rathnayake, A.D., Zheng, J., Kim, Y., *et al.* 3C-like protease inhibitors block coronavirus replication in vitro and improve survival in MERS-CoV-infected mice. *Sci. Transl. Med.* **12(557)**, eabc5332 (2020).
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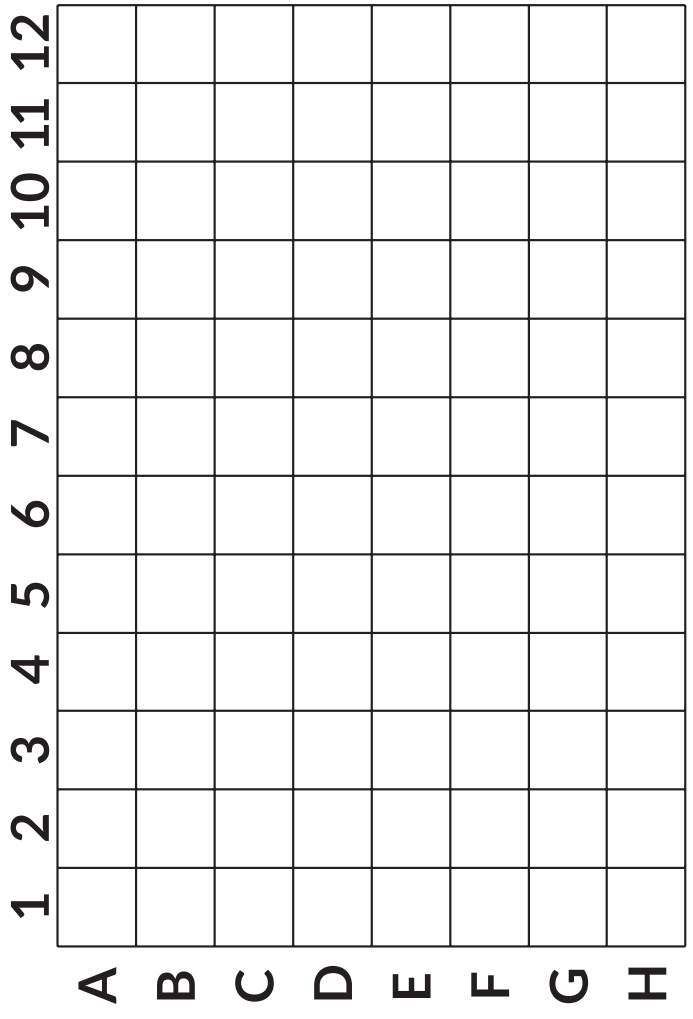

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.