# ACE2 Inhibitor Screening Assay Kit

Item No. 502100

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Customer Service 800.364.9897
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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 |
|---|---|---|---|
| 502101 | ACE2 Assay Buffer (10X) | 1 vial/10 ml | -20°C |
| 502102 | ACE2 Enzyme (human, recombinant) | 1 vial/20 µl | -80°C |
| 502103 | ACE2 Substrate (Mca-APK (Dnp)) | 1 vial/150 µl | -20°C |
| 502104 | ACE2 Inhibitor (MLN-4760) | 1 vial/40 µl | -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

**Fax:** 734-971-3640

**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 320 and 405 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. An orbital microplate shaker
4. A source of ultrapure water is recommended. Pure water - glass-distilled or deionized water - may not be acceptable *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Microcentrifuge tubes

# INTRODUCTION

## Background

Angiotensin-converting enzyme 2 (ACE2) is a carboxypeptidase and homolog of ACE1 that is encoded by *ACE2* in humans.[1,2] It is a type I transmembrane protein composed of a cytoplasmic tail and an extracellular domain containing an HEMGH zinc-binding motif, which exhibits carboxypeptidase activity.[1] ACE2 is expressed in vascular endothelial cells where it catalyzes the conversion of angiotensin II to the vasodilatory peptide angiotensin 1-7 to regulate systemic blood pressure and angiotensin I to angiotensin 1-9, a peptide that counter-regulates the function of angiotensin II.[1-3] It is also expressed in the epithelial cells of the kidney, heart, lung, small intestine, and liver and has roles in fluid homeostasis, cardiac contractility, and amino acid absorption, as well as the prevention of pulmonary fibrosis and hypertension. ACE2 also acts as a functional receptor for severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 to facilitate viral entry into host cells.[4,5] Inhibitors of the ACE2 and SARS-CoV-2 interaction may be beneficial against viral infection in the treatment of COVID-19. However, such inhibitors should not affect ACE2's carboxymonopeptidase activity, as doing so could exacerbate COVID-19 comorbidities as seen in *Ace2* knockout mouse studies. *Ace2*$^{-/-}$ mice exhibit increased angiotensin II-induced hypertension, susceptibility to atherosclerotic plaques, myocardial dysfunction, and insulin resistance compared with wild-type mice.[6]



## About This Assay

Cayman's ACE2 Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for testing potential inhibitors of human ACE2. The assay uses an ACE2-specific fluorogenic substrate, Mca-APK (Dnp). ACE2 cleaves this substrate generating free Mca, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 320 and 405 nm, respectively. The potent and reversible ACE2 inhibitor MLN-4760 is included as a positive control. Human ACE2 plays an important role in regulating blood pressure. It is also known to bind SARS-CoV-2 spike. This assay can be used as a counter-screen for potential drug candidates to ensure they do not interfere with the normal physiological functions of ACE2.

# PRE-ASSAY PREPARATION

## Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted ACE2 Assay Buffer at a concentration 20X the desired final assay concentration (*e.g.*, for 1 µM final assay concentration, a 20 µM stock should be made). This solution may contain up to 100% MeOH, 20% EtOH, or 1% DMSO or dimethyl formamide (DMF). The final concentration of organic solvents in the assay will then be ≤5% MeOH, <1% EtOH, or <1% DMSO or DMF (see 'Effects of Solvents' on page 17).

## Reagent Preparation

1. **ACE2 Assay Buffer (10X)**

   Mix 2 ml of ACE2 Assay Buffer (10X) (Item No. 502101) with 18 ml of ultrapure water to make 20 ml of ACE2 Assay Buffer (1X). This volume of ACE2 Assay Buffer (1X) is sufficient for 96 reactions. Scale up, if needed, for diluting test compounds. The ACE2 Assay Buffer (1X) should be discarded if not used within the same day. Unused ACE2 Assay Buffer (10X) should be re-frozen and stored at -20°C where it will be stable for at least one month.

2. **ACE2 Substrate (Mca-APK (Dnp))**

   This vial contains ACE2 Substrate (Mca-APK (Dnp)) (Item No. 502103) in 0.067% ammonium hydroxide. Mix 50 µl ACE2 Substrate (Mca-APK (Dnp)) with 450 µl ACE2 Assay Buffer (1X). This volume of diluted ACE2 Substrate (Mca-APK (Dnp)) is sufficient for 40 reactions. The diluted substrate will be stable at room temperature for two hours. If all of the ACE2 Substrate (Mca-APK (Dnp)) will not be used at one time, aliquot the undiluted substrate and store at -20°C where it will be stable for at least one month.

3. **ACE2 Enzyme (human, recombinant)**

   ACE2 Enzyme (human, recombinant) (Item No. 502102) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 5 µl of ACE2 Enzyme (human, recombinant) with 495 µl ACE2 Assay Buffer (1X). The diluted ACE2 Enzyme is sufficient for 40 reactions. It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme is stable when stored on ice for 1 hour. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

4. **ACE2 Inhibitor (MLN-4760)**

   This vial contains 40 µl of 2 mM ACE2 Inhibitor (MLN-4760) (Item No. 502104) in DMSO, which can be used as a positive control. Mix 10 µl of ACE2 Inhibitor with 90 µl ACE2 Assay Buffer (1X) to make a 200 µM stock solution. Then mix 10 µl of the 200 µM ACE2 Inhibitor stock solution with 90 µl ACE2 Assay Buffer (1X) to make a 20 µM working solution. If all of the ACE2 (MLN-4760) Inhibitor 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 ACE2 Inhibitor (MLN-4760), 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, below.

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

BW - Background Wells
A - 100% Initial Activity Wells
PC - Positive Control Wells
1-29 - Inhibitor Wells

**Figure 1. Sample plate format**



### Pipetting Hints

- It is recommended that an 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 diluted assay buffer in the assay.
- All reagents should be prepared as described above. All reagents except the enzyme should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- 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 320 nm and an emission wavelength of 405 nm.

## Performing the Assay

1. **Background Wells**: add 85 µl of ACE2 Assay Buffer (1X) and 5 µl of solvent to three wells. Use the same solvent concentration used for the unknown inhibitor and the positive control, ACE2 Inhibitor (MLN-4760). If different solvents are to be assayed at the same time, separate sets of background wells should be run for each solvent.
2. **100% Initial Activity Wells**: add 75 µl of ACE2 Assay Buffer (1X), 10 µl ACE2 Enzyme, and 5 µl of solvent to three wells. Use the same solvent concentration used for the unknown inhibitor and the positive control, ACE2 Inhibitor (MLN-4760). If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent.
3. **Inhibitor/Positive Control Wells**: add 75 µl of ACE2 Assay Buffer (1X), 10 µl of diluted ACE2 Enzyme, and 5 µl of unknown inhibitor or the 20 µM positive control, ACE2 Inhibitor (MLN-4760), working solution to three wells. *NOTE: To determine an $IC_{50}$ value for an inhibitor, multiple concentrations of the inhibitor should be tested in the assay.*
4. Initiate the reactions by adding 10 µl of ACE2 Substrate (Mca-APK (Dnp)) to all the wells being used. Mix by pipetting or placing on a plate shaker.
5. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for 30 minutes at room temperature, protected from light.
6. Remove the plate cover and read the plate with an excitation wavelength of 320 nm and an emission wavelength of 405 nm.



# 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:

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

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

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 human ACE2 by ACE2 Inhibitor (MLN-4760) 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.[7]

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

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 ACE2 Inhibitor Screening Assay Kit was determined to be 0.96.

**Sample Data:**

The data shown here is an example of the 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.

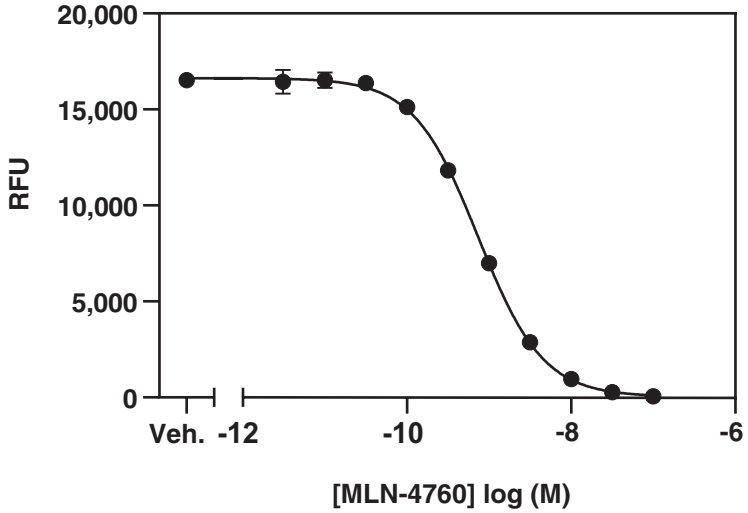



**Figure 2. Inhibition of ACE2 by ACE2 Inhibitor (MLN-4760).** Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity. The $IC_{50}$ value of ACE2 Inhibitor (MLN-4760) is 0.74 nM.

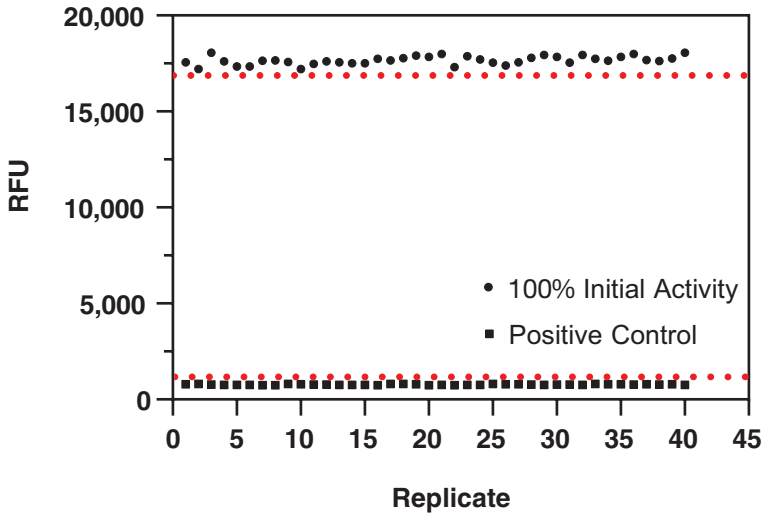



**Figure 3. Typical Z′ data for the ACE2 Inhibitor Screening Assay Kit.** Data are shown from 40 replicates each for vehicle control (Veh.), which represents 100% initial activity, and positive control wells prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.96. The red dotted 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% for MeOH, <1% for EtOH, and <1% for DMSO and DMF. A titration of organic solvents showed that the signal decreases with increasing DMSO, DMF, and EtOH concentration so the proper vehicle control should be included in the assay.

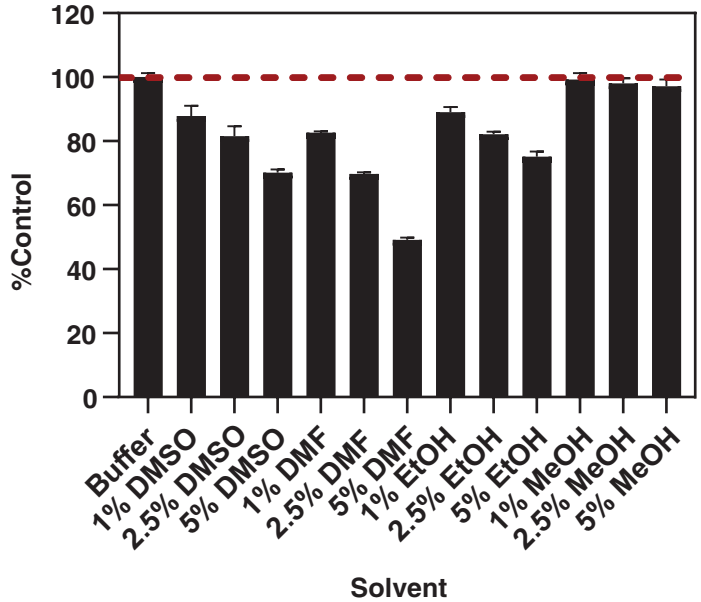



**Figure 4. The effect of solvent on the readout of ACE2 activity.** The data are shown as the mean ± standard deviation for triplicate reactions containing the indicated concentration of solvents.

**Precision:**

Intra-assay precision was determined by analyzing 24 measurements of the background and a vehicle control on the same day. The intra-assay coefficients of variation were 2.9 and 3.1%, respectively. The intra-assay coefficient of variation for the $IC_{50}$ value of seven inhibition curves performed on the same day was 3.3%.

Inter-assay precision was determined by analyzing inhibition with ACE2 Inhibitor (MLN-4760) in five separate assays on different days. The inter-assay coefficient of variance for the $IC_{50}$ value was 11.8%.



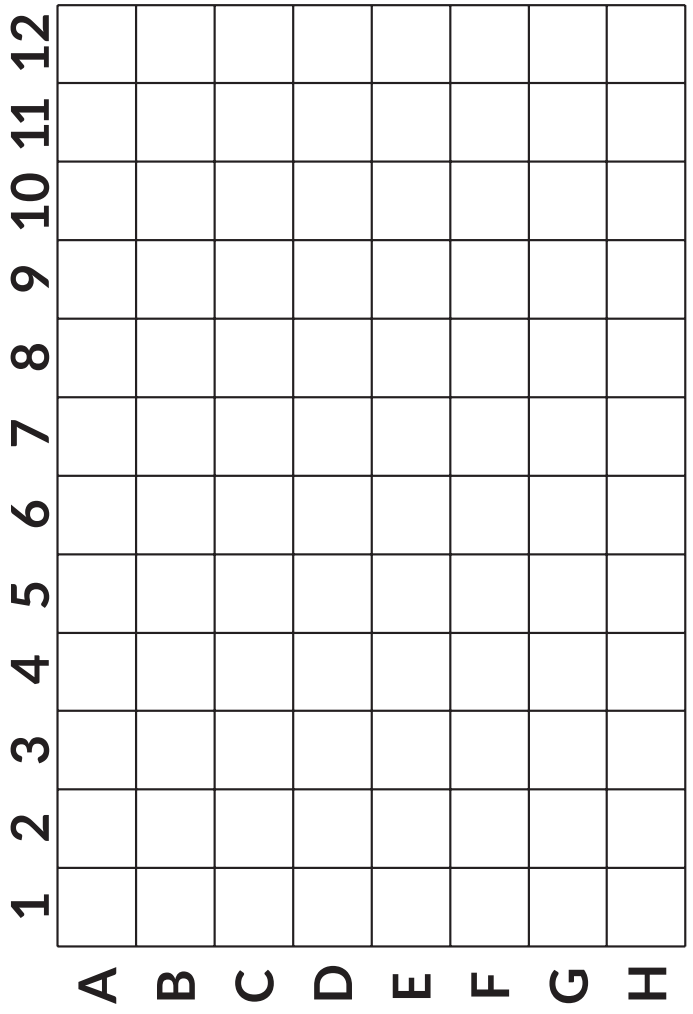
# RESOURCES

## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Erratic values; dispersion of duplicates/triplicates | A. Poor pipetting/technique<br>B. Bubble in the well(s) | A. Be careful not to splash the contents of the wells<br>B. Carefully tap the side of the plate with your finger to remove bubbles |
| No fluorescence detected above background in the inhibitor wells | A. Enzyme or substrate was not added to the well(s)<br>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)<br>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<br>B. The compound is not an inhibitor of the enzyme | Increase the compound concentration and re-assay |

## References


1. Perlot, T. and Penninger, J.M. ACE2 - from the renin-angiotensin system to gut microbiota and malnutrition. *Microbes Infect.* **15(13)**, 866-873 (2013).
2. Santos, R.A.S., Sampaio, W.O., Alzamora, A.C., *et al.* The ACE2/angiotensin-(1-7)/MAS axis of the renin-angiotensin system: Focus on angiotensin-(1-7). *Physiol. Rev.* **98(1)**, 505-553 (2018).
3. Ocaranza, M.P., Moya, J., Barrientos, V., *et al.* Angiotensin-(1—9) reverses experimental hypertension and cardiovascular damage by inhibition of the angiotensin converting enzyme/Ang II axis. *J. Hypertens.* **32(4)**, 771-783 (2014).
4. Hoffmann, M., Kleine-Weber, H., Schroeder, S., *et al.* SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* **181(2)**, 271-280 (2020).
5. Gurwitz, D. Angiotensin receptor blockers as tentative SARS-CoV-2 therapeutics. *Drug Dev. Res.* **81(5)**, 537-540 (2020).
6. Jia, H., Yue, X., and Lazartigues, E. ACE2 mouse models: A toolbox for cardiovascular and pulmonary research. *Nat. Commun.* **11(1)**, 5165 (2020).
7. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. *J. Biomol. Screen.* **4(2)**, 67-73 (1999).

## 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.