SARS-CoV-2 PLpro Activity Assay Kit (96 Rx) for use with Cellular Lysates

Product No: MP-PLpro-001
Lot No: 071024
vs. 071024

Note: Please follow approved biosafety protocols when using the assay kit in biosafety laboratories.

Contents and Storage

Reagent	Description	Concentration	Amount	Final concentration	Storage
Max 1	High RFU calibrator	1x	100 µl	1x	-20 °C
Max 2	Medium RFU calibrator	1x	100 µl	1x	
Min	Minimum RFU calibrator	1x	100 µl	1x	
Pun74-R110	Rhodamine110 labeled PLpro peptide in 100% DMSO	50 µM	10 µl	100 nM	
S11-R110	Rhodamine110 labeled caspase peptide in 100% DMSO	500 µM	10 µl	1 µM	
DTT	Dithiothreitol	1 M	100 µl	5 mM	
Assay Buffer	PLpro / caspase assay buffer	10x	5 mL	1X	CRT
Lysis Buffer	Mesa Photonics proprietary	2X	5 mL	1X	
Plate	Black 96-well plate		1		

Upon kit arrival, remove Assay Buffer and Lysis Buffer from the kit and store at room temperature. Store the other kit components at -20 °C.

General Guidelines

This Instructions for Use provides general guidelines for preparing lysates from SARS-CoV-2 infected VeroE6 cells and monitoring PLpro activity with the SARS-CoV-2 PLpro Activity Assay Kit. Both peptides can also be used to monitor cleavage in whole live cells, as described in the Application Note # 3 on Mesa Photonics website.

Note: The assay has been validated for use with VeroE6 cells (ATCC, Manassas, VA, USA, Cat# CRL-1586) and Vero-CCL81 infected with the SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, Manassas, VA, USA). The assay is expected, but not proven, to perform with other SARS-CoV-2 isolates. The performance of the peptide Pun74-R110 in lysates from cells other than VeroE6 is unknown.

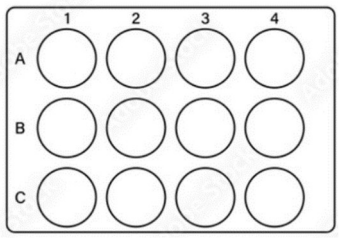
Additional Materials Required

- Tissue culture laboratory for growing and infecting cells—lysates are not provided in this kit.
- Fluorescence plate reader
- Sterile microcentrifuge tubes
- Appropriate pipettes and tips
- Microcentrifuge for quick spin

A. Cell Growth and Infection

1. Plate Vero cells in a 12-well plate according to established protocols.
2. When the cells have reached ~ 90% confluency (approximately 300,000 cells/well), use established protocols to infect with
    a. 0.1 – 0.01 MOI of SARS-CoV-2
    b. 0 MOI control

An example of a plate setup is shown below



Row A, wells 1-4	0.1 MOI
Row B, wells 1-4	0.01 MOI
Row C, wells 1-4	Uninfected control

Cells in column 1-3 are used to measure protease activities, cells in column 4 for determining cell numbers or determining cell health according to preferred methods (e.g., counting live cells with Tryptan blue, Alamar Blue staining etc.).

3. Incubate the infected cells for 12-24 hours at 37 °C in a humidified incubator
4. Prepare SARS-CoV-2 PLpro reagents as described in **Section B**
5. Remove media from wells
6. Wash cell monolayers 3X with 100 mM HEPES (room temperature)
7. Prepare cells in column 4 according to preferred protocol
8. Immediately before measuring protease activities, add 100 µL Lysis Buffer directly to each well in column 1-3
9. Let the lysate sit for 5 minutes. Do not let lysate sit for more than 15 minutes
10. Pipet up and down and combine lysates from **Row A, wells 1-3** in a microcentrifuge tube (Vial A). Repeat for **Row B** (Vial B) and **Row C** (Vial C)
11. Add 25 µL lysate to wells of the provided black 96-well plate

    An example of an experimental setup is shown in Section B

Notes:

Remaining lysates can be frozen at -80 C for at least 1 week without significantly losing protease activities. Performance after storage for longer than 1 week has not be tested.

B. Protease Activity Assays

1. Start plate reader and open protocol template configured in Section C
2. Thaw Pun74, S11, and DTT at room temperature, gently vortex to mix, spin to collect droplets

IMPORTANT: Pun74 and S11 are dissolved in 100% DMSO – be sure contents have thawed before use

3. **Prepare Complete Assay Buffer** (fresh for each experiment)
   100 µl 10x Assay Buffer
   10 µl 1M DTT
   890 µl H_20
   1000 µl
4. **Prepare Substrate Solutions** (fresh; just before cells are ready to lyse)

   For detection of caspase activity
   1 µl S11-R110
   249 µl Complete Assay Buffer
   250 µl

   For detection of PLpro activity
   1 µl Pun74-R110
   249 µl Complete Assay Buffer
   250 µl

5. To wells containing 25 µL lysate (see Section A, step 11) add:

   25 µL 2X **S11** Solution to wells in column 1-3

   25 µL 2X **Pun74** Solution to wells in column 4-6

   An example of a plate setup is shown below

	Row / Column	1	2	3	4	5	6
Vial A: wells A 1-9 → 25 µL	**A**						
Vial B: wells B 1-9 → 25 µL	**B**						
Vial C: wells C 1-9 → 25 µL	**C**						

6. Insert plate into plate reader and read immediately

C. Configure Protocol and Read in Plate Reader

Mesa Photonics protease assays are “turn on” assays, i.e., the fluorescence increases over time, and some readers will auto-adjust the photomultiplier tube (PMT) settings from one measurement to the next. If running progress curves, data acquired from different timepoint reads cannot be compared.

It is therefore recommended to monitor the reaction progress in kinetic mode using one PMT setting defined in a protocol rather than taking several separate measurements in endpoint mode.

However, if the reader software allows to manually input a gain number (e.g., 100V), endpoint mode reads with the same PMT setting can be performed.

Establish PMT settings on Plate Reader

The peptides provided in this kit are extremely bright and easily oversaturate the PMT, resulting in OVFLW reads. It is recommended to use the provided calibrators to adjust the PMT settings using Max and Min controls provided before running experiments:

1. Thaw vials labeled Max1, Max2, and Min
2. Pipet 50µL of each into a well of the provided 96 well plate
3. Adjust PMT settings according to instrument manufacturer’s instructions
   Generally, PMT on LOW correspond to voltages of ~ 100V and avoid oversaturation
4. If required, reduce lamp energy in the setup protocol
5. Max 1 RFU adjustment allows monitoring RFU from DEVD peptide cleavage
6. Max 2 RFU adjustment allows monitoring RFU from Pun74 peptide cleavage
7. Min RFU determines the S/B that can be expected with each PMT setting

Note: Calibrator solutions can be returned to vials and frozen for future use.

A protocol from a Synergy H1 monochromator-based plate reader (BioTek/Agilent) is given below as an example.

Plate Type	Cliniplate 96 flat bottom black
Well Selection	Runtime
Set Temperature	Setpoint 30°C
Preheat before moving to next step	
Start Kinetic	Runtime 1:00:00 (HH:MM:SS), Interval 0:05:00, 12 Reads
Shake	Linear: 0:03 (MM:SS)
Frequency:	567 cpm (3 mm)
Filter Set 1	Excitation: 485, Emission: 528
Optics:	Top
Gain:	**100**
Light Source:	Xenon Flash
Lamp Energy:	**High**
Read Speed:	Normal
Delay:	100 msec
Measurements/Data Point:	10
Read Height:	7 mm
End Kinetic	

Note: If OVRFLW reads are obtained despite setting the gain according to the Max1 and Max2 calibrators, one of the following steps can be taken:

- Stop the read, lower the gain setting and start a new read.
- Remove 25 µl of the well contents and add 25 µl assay buffer
- Repeat the experiment using a final concentration of 50 nM Pun74 and 500 nM S11.

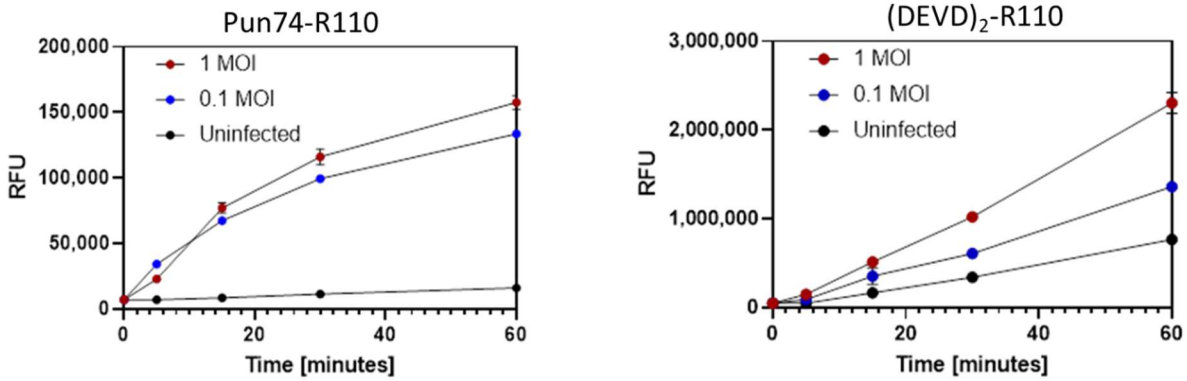
D. Guidelines for Data Analysis

1. Substrate depletion may lead to a flattening of the progress curve after 30 minutes (see figure below, left).
   ➔ determine the Relative Fluorescence Units (RFU) from the time point at which the reaction progress is still linear.
2. The reaction may have progressed before the first read was taken
   ➔ Subtract the RFU from the uninfected control from the experimental data point at the same timepoints to calculate delta RFU
3. If comparing data from different treatment groups, normalize the data for the number of live cells that were determined using independent protocols (see Section A).

Notes:

S11 ($(DEVD)_2$-R110) is cleaved by caspases that are activated in cells preparing to undergo apoptosis. Increases in fluorescence will therefore correlate with MOI, but are also apparent in lysates from uninfected control cells.

An example of raw data from an experiment performed according to this protocol is shown below.



Left: The RFU from cleavage of Pun74-R110 in lysates from SARS-CoV-2 infected cells are significantly above the background of RFU from lysates of uninfected cells. The reaction progress is linear for ~ 20 minutes. PLpro activities in lysates from cells infected with 1 MOI and 0.1 MOI do not significantly differ.

Right: Increases in RFU from cleavage of $DEVD_2$-R110 (S11 supplied in this kit) scale with MOI and are approximately linear over 60 minutes. Increases in RFU are also apparent in lysates from uninfected cells, which may be due to stress in tissue cultured cells after > 24 hours of incubation and preparation for cell death, i.e., activation of caspase that cleave DEVD amino acid sequences.