SensoLyte® 520 SARS-CoV-2 Papain-like Protease/ Deubiquitinase Activity Assay Kit *Fluorimetric*

Revision number: 1.0 *Last updated:15JAN2025*

Catalog #	AS-72274
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect SARS-CoV-2 Papain-like protease activity.
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Papain-like Protease Deubiquitination substrate Ex/Em=490 nm/520 nm upon cleavage	400 µM, 50 µL
Component B	Rh110 fluorescence reference standard, Ex/Em=490 nm/520 nm	400 µM, 15 µL
Component C	2X Assay Buffer	25 mL
Component D	Inhibitor (GRL0617)	10 mM, 10 µL

Other Materials Required (but not provided)

- Recombinant SARS-CoV-2 Papain-like protease source: Active enzyme (R&D systems, Cat#E611-050)
- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components at -20°C.
- Protect Components A and B from light and moisture.
- Component C can be stored at room temperature for convenience.

Introduction

SARS-Cov-2 Papain-like Protease (PL^{pro}) is encoded as non-structural protein 3 (nsp3) and it is involved in the cleavage of the viral polyprotein[1-3]. This enzyme hydrolyzes ubiquitin chains which are important in the inflammation process and also removes interferon-stimulated gene 15 (ISG15) modifications from proteins to reverse antiviral responses [1-5] (Figure 1). As an essential enzyme in the process of SARS-CoV-2 replication and infection of the host, PLpro has been an attractive drug target for the coronavirus inhibitor development [2, 6].

The SensoLyte® 520 SARS-CoV-2 Papain-like Protease Deubiquitination Assay Kit employs a fluorescence peptide substrate for the detection of deubiquitnation activity. When active Papain-like Protease cleaves the substrate, it results in an increase of bright green fluorescence at excitation/emission = 490 nm/520 nm. This assay can detect as low as 7.8 ng/mL active Papain-like Protease in the sample. This kit can be used to detect enzyme activity in purified enzyme preparations, biological samples and can also be applied for the compound screening.

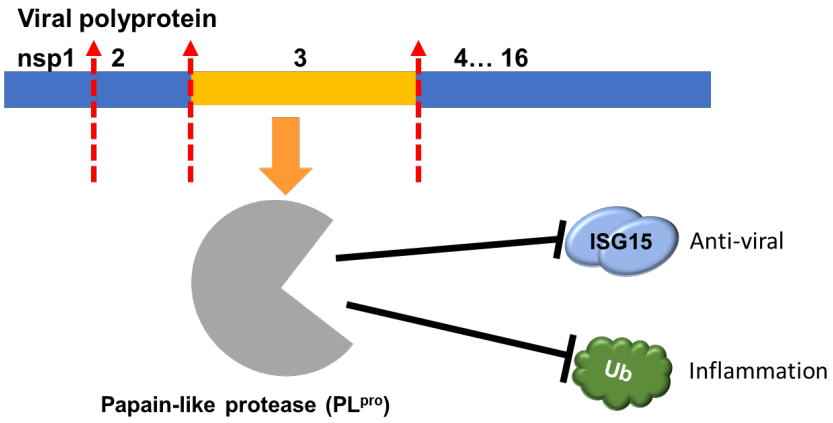

Figure 1. SARS-CoV-2 Papain-like protease activities. SARS-CoV-2 Papain-like protease cleaves viral polyprotein. It has been shown to suppress the host innate immune responses by its ability for deubiquitination and deISGylation.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screening Papain-like protease inhibitors using a purified enzyme.

1. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

1.1 1X assay buffer: Add 10 ml of 2X Assay Buffer (Component C) to 10 mL deionized water. Use this 1X assay buffer for all subsequent steps. If not using the entire plate, adjust the amount of assay buffer to be diluted accordingly.

1.2 520 Papain-like Protease substrate solution: Dilute substrate (Component A) 100-fold in assay buffer. Refer to Table 1.

Table 1. Papain-like protease substrate solution for one 96-well plate (100 assays).

Components	Volume
Papain-like protease substrate (100X, Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

1.3 SARS-CoV-2 Papain-like protease diluent: Dilute the enzyme to an appropriate concentration in assay buffer (Component C).

Note: ***Recommended amount of enzyme is 50 ng/well.*** Prepare enzyme diluents immediately before use. Do not vortex enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause the enzyme denaturation. Store the enzyme solution on ice.

1.4 Inhibitor (GRL0617): Dilute the 10 mM inhibitor solution (Component D) 1:50 in assay buffer. The diluted GRL0617 solution has a concentration of 200 µM. Add 10 µl of the diluted GRL0617 into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. The suggested volume of enzyme solution for a 96-well plate is 40 µL/well and test compound is 10 µL/well.

2.2 Simultaneously set up the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains Papain-like protease and GRL0617.
- Vehicle control contains Papain-like protease and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Use the assay buffer to bring the total volume of all controls to 50 µL.

3. Run the enzymatic reaction.

3.1 Add 50 µL of Papain-like protease substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction at 37ºC for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

3.3 For methods of data analysis: Refer to Appendix I.

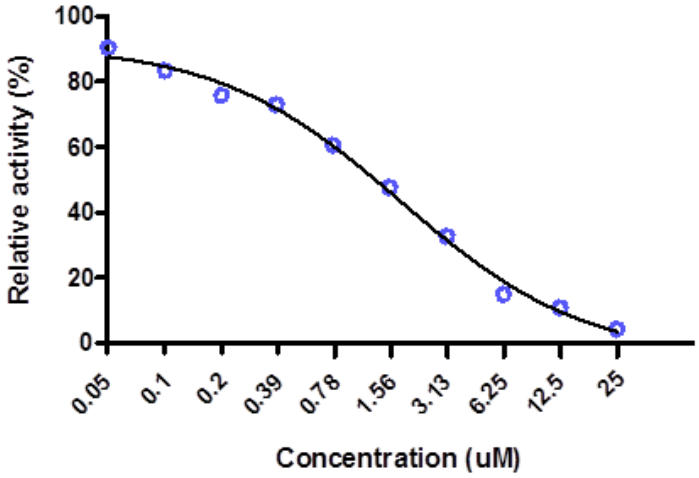

Figure 2. Inhibition of Papain-like protease activity by GRL0617 as measured with SensoLyte® 520 SARS-CoV-2 Papain-like Protease Deubiquitination Assay Kit.

Protocol B. Measuring Papain-like protease activity in biological samples.

1. Prepare SARS-CoV-2 Papain-like protease containing biological samples.

1.1 Prepare sample from cell culture medium:

- Collect medium from culture.
- Spin the medium sample for 10-15 min. at 1,000X g, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare cell lysates:

- Cells are collected by centrifugation at 500 X g for 10 min.
- Add an appropriate amount of cold lysate buffer to cell pellet. Collect the cell suspension to a microcentrifuge tube.
- Incubate the cell suspension on ice for at least 10 min.
- Pipette the cell suspension up and down for 5 times.
- Centrifuge the cell suspension for 5 min. at 10,000 X g, 4°C. Collect the supernatant and store at -70°C until use.

Note: CHAPS concentration below 1% is compatible with this assay. Other detergents used in lysate buffer formulation may affect the assay reaction.

2. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

2.1 The Papain-like protease substrate solution: Dilute substrate (Component A) 100-fold in 2X assay buffer (Component C). Refer to Table 1. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 1. Papain-like protease substrate solution for one 96-well plate (100 assays)

Components	Volume
Papain-like protease substrate (Component A)	50 µL
2X assay buffer (Component C)	4.95 mL
Total volume	5 mL

2.2 Papain-like protease diluent: If using purified Papain-like protease as a positive control, it must be diluted to an appropriate concentration in assay buffer (Component C).

Note: ***Recommended amount of enzyme is 50 ng/well.*** Prepare enzyme diluents immediately before use. Do not vortex enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause the enzyme denaturation. Store the enzyme solution on ice.

3. Set up enzymatic reaction.

3.1 Add 5-50 µL of Papain-like protease containing biological sample.

3.2 Set up the following control wells at the same time, as deemed necessary:

- Positive control contains purified Papain-like protease.
- Substrate control contains assay buffer.

3.3 Using the assay buffer, bring the total volume of all controls to 50 µL.

4. Run the enzymatic reaction.

4.1 Add 50 µL of Papain-like protease substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure fluorescence signal:

4 For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.

5 For end-point reading: Incubate the reaction at 37 ºC for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 For methods of data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other

wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).

- For kinetics analysis:
  - Plot data as RFU versus time for each sample. To convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, K_m, K_i, etc.
- For endpoint analysis:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- Rh110 Fluorescence reference standard: Dilute 400 µM Rh110 fluorescence reference standard (Component B) to 4 µM in assay buffer. Do 2-fold serial dilutions to get concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 µM, and include 0 µM as an assay buffer blank. Add 50 µL/well of these serially diluted fluorescence reference solutions.
- Add 50 µL/well of the diluted Papain-like protease substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The Papain-like protease substrate solution is added to the fluorescence reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of fluorescence reference standard are 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313 and 0 µM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

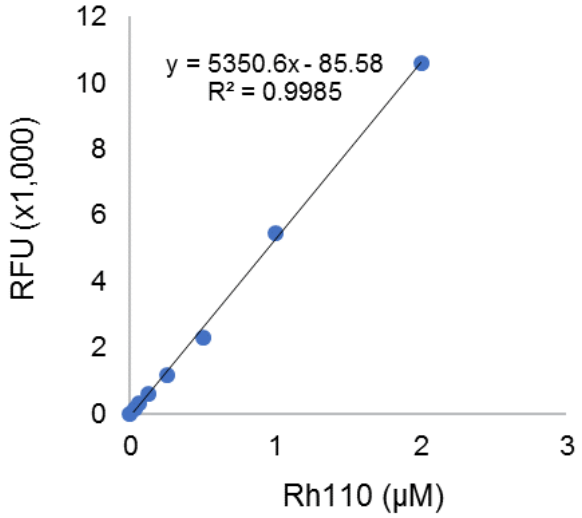

Figure 3. Rh110 fluorescence reference standard. Rh110 fluorescence was serially diluted in assay buffer, containing Papain-like protease substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (SpectraMax M^{5e}, Molecular Devices).

References

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