SensoLyte® 520 SARS-CoV-2 3CL Protease Activity Assay Kit **Fluorimetric**

Revision number: 1.0 *Last updated: 28/JUL/2020*

Catalog #	AS-72262
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect SARS-CoV-2 3CL 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	Hilyte™ Fluor-488/QXL™ 520 3CL Protease substrate Ex/Em=490 nm/520 nm upon cleavage	1 mM, 50µL
Component B	Hilyte™ Fluor-488 fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 10 µL
Component C	Recombinant SARS-CoV-2 3CL Protease	0.1 mg/mL, 50 µL
Component D	2X Assay Buffer	20 mL
Component E	Inhibitor (GC 376)	10 µM, 10 µL

Other Materials Required (but not provided)

- 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, except for Component C
- Store Component C at -80°C
- Protect Components A and B from light and moisture.

Introduction

SARS-CoV-2 3-chymotrypsin-like protease (3CL protease, also called the main protease) is essential for processing the polyproteins that are translated from the viral RNA[1, 2]. It is involved in the transcription and replication during the viral infection[3, 4]. 3CL protease cleaves the replicase 1ab at least 11 cleavage sites producing proteins that are assembled into the virion[2] (Figure 1). The advantage of choosing 3CL protease as the SARS-CoV-2 virus drug target is that the protein structure is well conserved across the *Coronaviridae* family[2, 5].

The SensoLyte® 520 SARS-CoV-2 3CL Protease Activity Assay Kit employs a fluorescence FRET peptide substrate for the detection of enzyme activity. The sequence of this substrate is based on the site-specific cleavage sequence for coronavirus 3CLpro enzymes. When active 3CL Protease cleaves the FRET substrate, it results in an increase of HiLyte Fluor™ 488 green fluorescence monitored at excitation/emission = 490 nm/520 nm. With superior fluorescence quantum yield and longer emission wavelength, the HiLyte™ Fluor 488/QXL® 520 based FRET peptide has less interference from the autofluorescence of test compounds and cellular components and provides better assay sensitivity. This assay can detect as low as 15.6 ng/mL active 3CL 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.

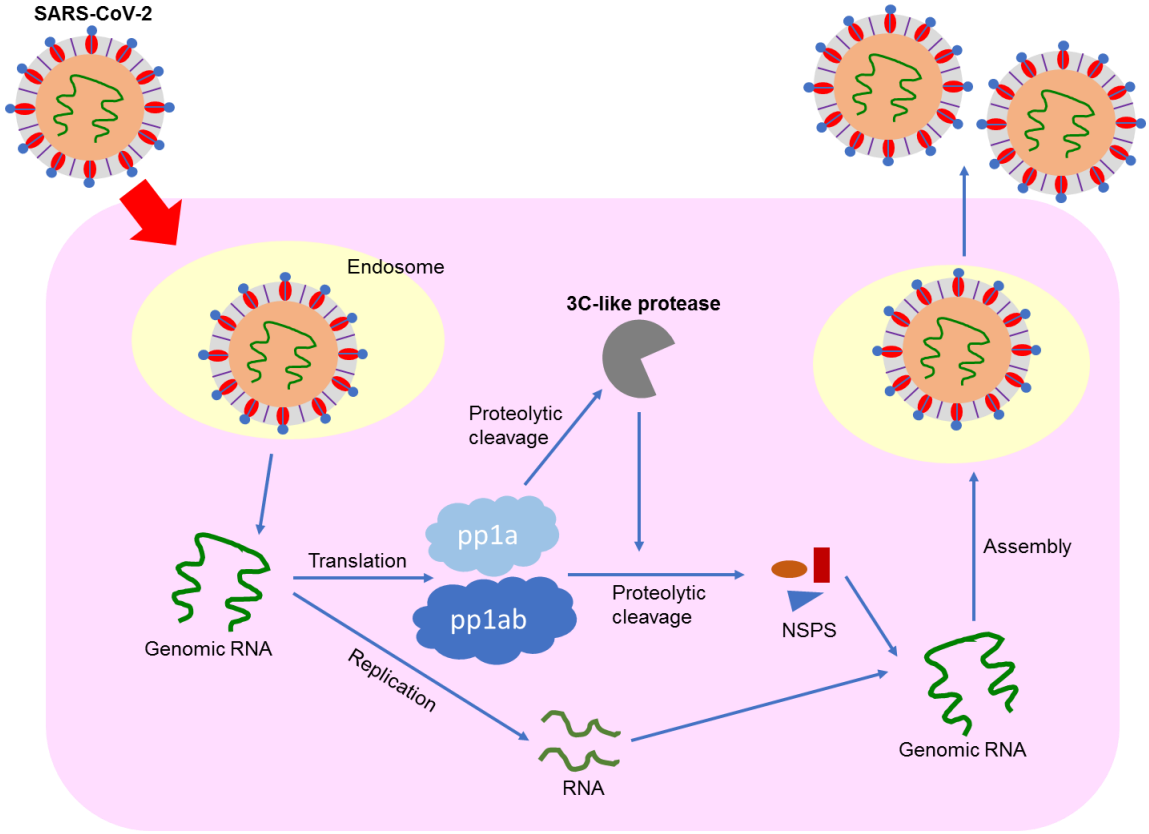

Figure 1. 3CL protease is involved in the transcription and replication during the viral infection [6].

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 3CL 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 D) to 10 mL of 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 3CL protease substrate solution: Dilute substrate (Component A) 100-fold in assay buffer. Refer to Table 1.

Table 1. 3CL protease substrate solution for one 96-well plate (100 assays).

Components	Volume
3CL protease substrate (100X, Component A)	50 μL
Assay buffer	4.95 mL
Total volume	5 mL

1.3 3CL protease diluent:

Dilute 3CL protease 80-fold in assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using an entire plate, adjust the amount of enzyme to be diluted accordingly.

Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store on ice.

1.4 Inhibitor (GC 376): Dilute the 10 μM inhibitor solution (Component E) 1:100 in assay buffer. The diluted GC 376 solution has a concentration of 100 nM. Add 10 μl of the diluted GC 376 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 3CL protease and GC 376.
- Vehicle control contains 3CL 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 3CL 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.

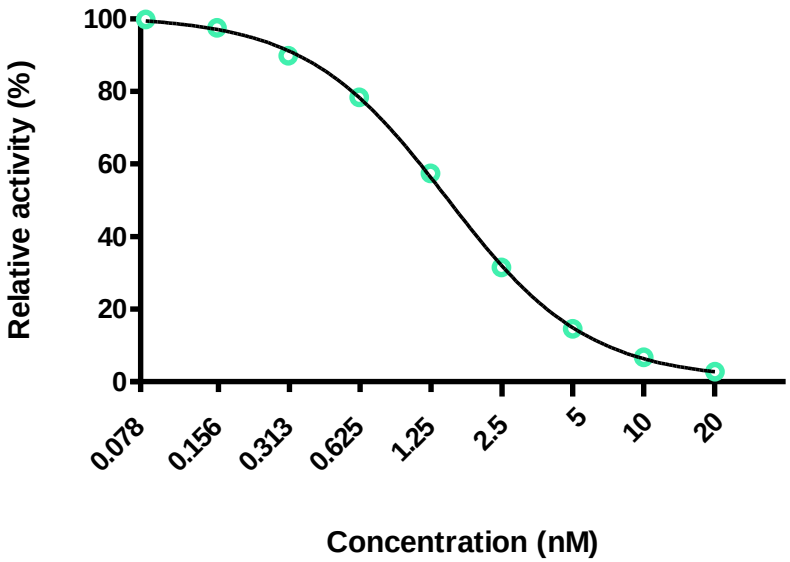

Figure 2. Inhibition of 3CL protease activity by GC 376 as measured with SensoLyte® 520 SARS-CoV-2 3CL Protease Activity Assay Kit.

Protocol B. Measuring 3CL protease activity in biological samples.

1. Prepare 3CL 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 assay 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.

2. Prepare working solutions.

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

2.1 Dilute 3CL protease substrate (Component A) 100-fold in 2X assay buffer. Refer to Table 1. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 1. 3CL protease substrate solution for one 96-well plate (100 assays).

Components	Volume
3CL protease substrate (100X, Component A)	50 µL
2X Assay buffer	4.95 mL
Total volume	5 mL

2.2 3CL protease diluent: If using purified 3CL protease as a positive control, do 100-fold dilution to the enzyme with 1X assay buffer (please refer to Protocol A, step1.1). Add 50 µl of the diluted enzyme into each of the positive control well.

Note: 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 3CL protease containing biological sample.

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

- Positive control contains purified 3CL 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 3Cl 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

- Hilyte™ Fluor-488 fluorescence reference standard: Dilute 1 mM Hilyte™ Fluor-488 fluorescence reference standard (Component B) to 10 μM in assay buffer. Do 2-fold serial dilutions to get concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156 μM, and include 0 μM as an assay buffer blank. Add 50 μL/well of these serially diluted Hilyte™ Fluor-488 reference solutions.
- Add 50 μL/well of the diluted 3Cl protease substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The 3CL protease substrate solution is added to the Hilyte™ Fluor-488 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 Hilyte™ Fluor-488 fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.

- The final concentrations of Hilyte™ Fluor-488reference standard are 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 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.

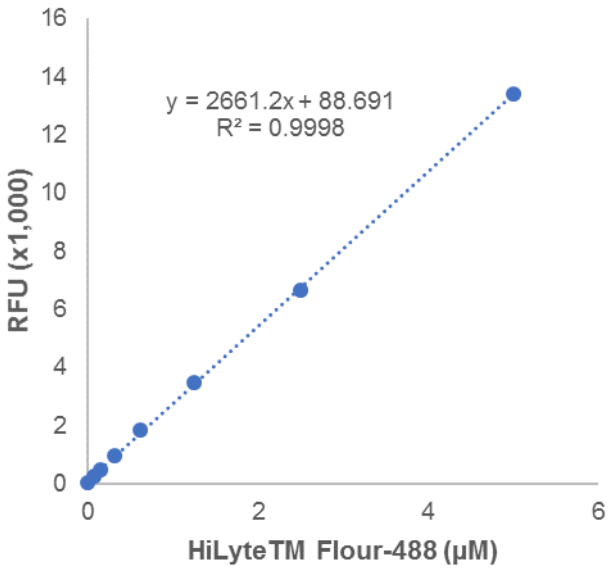

Figure 3. Hilyte™ Fluor-488 fluorescence reference standard. Hilyte™ Fluor-488 was serially diluted in assay buffer, containing 3CL protease substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (SpectraMax M5e, Molecular Devices).

References

1. Hilgenfeld, R. et al, *FEBS J* **281**, 4085 (2014).
2. Zhang, L. et al, *Science*. **368**, 409 (2020).
3. Thiel, V. et al, *J Gen Virol* 84, 2305 (2003).
4. Macchiagodena, M. et al, *Chem Phys Lett* **750**, 137489 (2020).
5. Chen, YW. et al, *F1000Res* 9, 129 (2020).
6. Mengist HM. et al, *Signal Transduct Target Ther* **5**, 67 (2020).