Description

The 3CL Protease (T21I, E166V) (SARS-CoV-2) Assay Kit is a 96-well homogeneous fluorogenic assay designed to measure the activity of T21I, E166V mutated 3CL Protease for screening and profiling applications, with no time-consuming washing steps. The kit contains enough purified 3CL Protease (T21I, E166V) (BPS Bioscience #101671), fluorogenic substrate, and 3CL Protease assay buffer for 100 enzyme reactions. 3CL inhibitor GC376 is also included as a control.

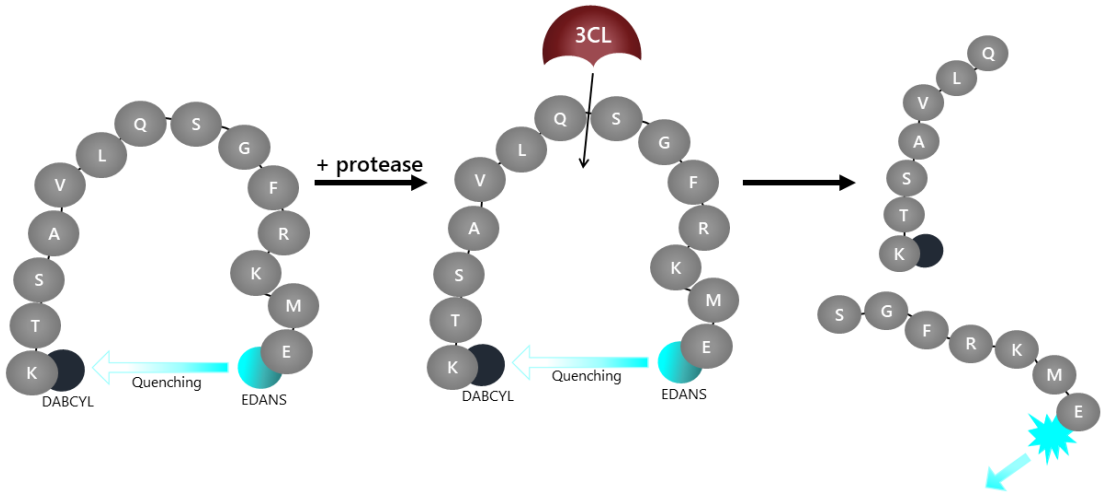

Figure 1: Illustration of the principle behind FRET-based 3CL protease assay.

The 3CL Protease Substrate is an internally quenched 14-mer fluorogenic (FRET) peptide (DABCYL-KTSAVLQSGFRKME-EDANS). When the donor (EDANS) and acceptor (DABCYL) fluorophores are in close proximity the energy emitted from EDANS is quenched by DABCYL (intact substrate). Upon proteolysis by 3CL, the peptide substrate is cleaved between the glutamine and serine residues to generate the highly fluorescent peptide fragment (SGFRKME-EDANS). The fluorescence intensity increases proportionally to the activity of 3CL. More information on the substrate, including MW and structure, can be found on our website (BPS Bioscience #79952).

Background

Coronaviruses (CoVs) cause respiratory and intestinal infections in humans and animals. The 3CL protease, also known as Main Protease (Mpro), plays a vital role in processing the polyproteins that are translated from the viral RNA. Protease inhibitors that can block viral replication are promising potential drug candidates for the treatment of patients suffering from COVID-19 infection.

T21I, E166V have been identified as mutations of interest for drug resistance.

Mutations

T21I, E166V

Applications

Study enzyme kinetics and screen small molecular inhibitors for drug discovery and High Throughput Screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
101671	3CL Protease (T21I, E166V) (SARS-CoV-2)*	30 µg	-80°C
79952	3CL Protease Substrate (10 mM)	50 µl	-80°C
79956	3CL Protease Assay Buffer	25 ml	-20°C
78013	GC376, MW = 507.5**	50 µg	-20°C
	0.5 M DTT	200 µl	-20°C
79685	Black, low binding microtiter plate	1	Room Temp.

** The concentration of protein is lot-specific and will be indicated on the tube containing the protein.*
***3CL inhibitor GC376 is provided as a control for 3CL inhibition.*

Materials Required but Not Supplied
Fluorescent microplate reader capable of reading λexc/λem=360 nm/460 nm

Stability
This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed.

Safety
This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Assay Protocol
All samples and controls should be tested in duplicate.

1. Just before use, dilute **0.5 M DTT** 500-fold in **3CL Protease Assay Buffer** to obtain a DTT concentration of 1 mM. This makes the **1x Assay Buffer**. Prepare enough DTT-containing buffer as required for the assay. Store the remaining stock **3CL Protease Assay Buffer** at -20°C.

2. Thaw **3CL Protease** (T21I, E166V) (SARS-CoV-2) on ice. Briefly spin the tube containing the enzyme to recover the full content of the tube.

   Note: 3CL Protease enzyme is sensitive to freeze/thaw cycles. Do not re-use the diluted enzyme.

3. Dilute **3CL Protease** (T21I, E166V) (SARS-CoV-2) in 1x Assay Buffer to 10 ng/µl. You need 30 µl/well.

   Note: The exact concentration and volume of enzyme is lot-specific and will be indicated on the tube. Calculate the required dilution from the information on the tube. It may be desirable to dilute the enzyme serially to avoid using large amounts of assay buffer for the dilution.

4. Add 30 µl of diluted **3CL Protease** (T21I, E166V) to the wells designated as "Positive Control", "Inhibitor Control" and "Test Inhibitor".

5. Add 30 µl of 1x Assay Buffer to the "Blank" wells.

6. Dilute the 50 µg vial of GC376 provided in 200 µl of 1x Assay Buffer to obtain a 500 µM solution. Add 10 µl of GC376 (500 µM) to the wells labeled "Inhibitor Control." Aliquot and store remaining solution at -80°C.

7. Prepare Test Inhibitor (10 µl/well): for a titration, prepare serial dilutions at concentrations 5-fold higher than the desired final concentrations. The final volume of the reaction is 50 µl.

   7.1. If the test inhibitor is soluble in water, prepare dilutions in 1x Assay buffer at concentrations 5-fold higher than the final desired concentrations. The 1x Assay Buffer is the Diluent Solution.

   OR

   7.2. If the Test Inhibitor is soluble in DMSO, dissolve in 100% DMSO at a concentration 100-fold higher than the highest desired concentration. Then make a 20-fold dilution in 1x Assay Buffer. The compound concentration is 5-fold higher than the final highest desired concentration and the concentration of DMSO is 5%.

   Prepare serial dilutions of the Test Inhibitor at concentrations 5-fold higher than the desired final concentrations using 5% DMSO in 1x Assay Buffer to keep the concentration of DMSO constant.

   For positive and negative controls, use 5% DMSO in 1x Assay Buffer so that all wells contain the same amount of DMSO (Diluent Solution).

   Note: The final concentration of DMSO in the assay should not exceed 1%.

8. Add 10 µl of Test Inhibitor to each well designated "Test Inhibitor".

9. Add 10 µl Diluent Solution to the "Blank" and "Positive Control" wells.

10. Preincubate for 30 minutes at Room Temperature with gentle agitation.

11. Dilute 40 µl of **3CL Protease Substrate** (10 mM) in 0.96 mL 1x Assay Buffer, to make a 400 µM solution. The final concentration of the 3CL Protease Substrate in the final 50 µl reaction is 80 µM.

12. Start the reaction by adding 10 µl of the diluted 3CL Substrate solution to <u>all</u> the wells.

13. Incubate for 1 hour at Room Temperature with gentle agitation.

Component	Blank	Positive Control	Test Inhibitor	Inhibitor Control
Diluted 3CL Protease (T21I, E166V) (10 ng/µl)	-	30 µl	30 µl	30 µl
1x Assay Buffer	30 µl	-	-	-
Diluted GC376 (500 µM)	-	-	-	10 µl
Test Inhibitor	-	-	10 µl	-
Diluent Solution	10 µl	10 µl	-	-
Incubate 30 minutes at Room Temperature				
Diluted 3CL Protease substrate	10 µl	10 µl	10 µl	10 µl
Incubate 1 hour at room temperature				
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**

14. Measure the fluorescence intensity in a microtiter plate-reading fluorimeter capable of excitation at 360 nm and detection of emission at 460 nm. The fluorescence intensity can also be measured kinetically.

    Note: GC376 and other 3CL protease inhibitors form reversible covalent modifications, thus IC_{50} values may increase with longer incubation times. "Blank" value should be subtracted from all other values.

Example of Assay Results

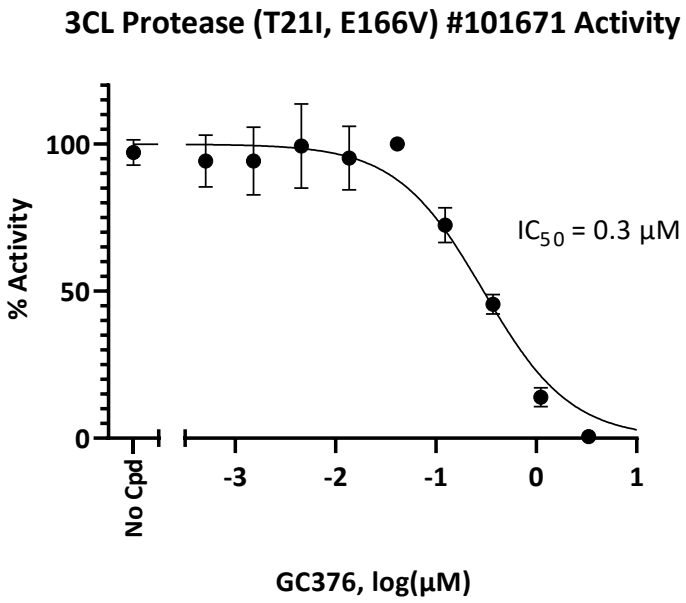

*Figure 2: Inhibition of 3CL Protease enzyme activity by increasing concentrations of GC376 (BPS Bioscience #*78013).
3CL Protease enzyme activity was measured in the presence of increasing concentrations of inhibitor GC376. Fluorescence intensity was measured using a Tecan fluorescent microplate reader. Results are expressed as percent of control activity (measured in the absence of GC376 and set at 100%).

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

General considerations

"Blank" Control: The "Blank" control is important to determine the background absorbance in the assay.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For further questions, please email support@bpsbioscience.com

References

1. Morse JS, *et al*., 2020, *Chem.Bio.Chem*. 21: 730-738.
2. Chi-Pang C, *et al*., 2011, *PLoS ONE* 6(11): e27228.
3. Iketani S, *et al*., 2023, Multiple pathways for SARS-CoV-2 resistance to nirmatrelvir. *Nature* 613, 558–564

Related Products

Products	*Catalog #*	*Size*
3CL Protease (B.1.1.529, Omicron Variant) (SARS-CoV-2)	101328	100 µg/1 mg
3CL Protease, Untagged (SARS-CoV-2) Assay Kit	78042	96 reactions/384 reactions
3CL Protease (T21I, S144A) (SARS-CoV-2) Assay Kit	78834	96 reactions
3CL Protease (T21I, A173V, T304I) (SARS-CoV-2) Assay Kit	78836	96 reactions
3CL Protease (T21I, A173V) (SARS-CoV-2) Assay Kit	78837	96 reactions
3CL Protease (T21I, T304I) (SARS-CoV-2) Assay Kit	78838	96 reactions
3CL Protease (P252L) (SARS-CoV-2) Assay Kit	78839	96 reactions
3CL Protease (SARS-CoV-2)	100823	50 µg/500 µg
3CL Protease (SARS-CoV-1) Assay Kit	78015	96 reactions
3CL Protease (MERS-CoV) Inhibitor Screening Assay Kit	78278	96 reactions