3CL Protease Activity Assay Kit

Kit I – Cat# J7510 (120 assays)
Kit II – Cat# J7511 (1,200 assays)

The kit measures the enzymatic activity of SARS-CoV-2 3CL protease (3CLPro), can be used for screening/profiling 3CLPro inhibitors. Kit I is sufficient for 120 X 50µl assays; Kit II is sufficient for 1,200 X 50µl assays. Optimized for rapidly and quantitatively measuring 3CLPro activity under room temperature.

Key Features:

- Optimized for room temperature operation, particularly useful for HTS assays
- Rapid and quantitative result within 2-3 hours
- Positive 3CLPro inhibitor PF07321332 included
- Large excess amount of buffer provided
- High concentration component stocks allowing custom assay development

Component Information

Components	**Quantity (Kit I, J7510)**	**Quantity (Kit II, J7511)**
10X GST-3CLPro (Enzyme)	4µM, 120µl	4µM, 1.2ml
100X Dabcyl-KTSAVLQSGFRKME-Edans (Substrate)	10mM in DMSO, 12µl	10mM in DMSO, 120µl
100X PF-07321332 (Inhibitor)	3.34mM in DMSO, 10µl	3.34mM in DMSO, 20µl
5X Assay Buffer	2X1.2ml	24ml

Required materials not included in the kit

1. 96-well black, clear flat bottom, non-binding microplate
2. Microplate reader detecting fluorescence at excitation/emission of 360/530 nm

Storage and Handling

Store all components at -80^{0}C; protect from light; avoid multiple freeze/thaw cycles.

Notes:

1. Aliquot the provided enzyme and substate if needed, snap freeze using liquid nitrogen and store under -80^{0}C.
2. Do not vortex components in the kit, including the Assay Buffer.
3. Diluted GST-3CLPro and the fluorogenic substrate in 1X Assay Buffer are stable for at least 4 hours under room temperature (*Figure A below*).

4. Use the kit to run a few test reactions to optimize plate reader settings to achieve an excellent signal/noise ratio.
5. When diluting 100X Dabcyl-KTSAVLQSGFRKME-Edans or chemical compounds, pipetting the stock solution into a tube bottom first, then adding room temperature 1X Assay buffer in once (no drop-wise addition) and immediately pipetting to dissolve the substrate or chemical compounds. Do not reverse the order by adding the substrate or compound stock into 1X Assay buffer as this may cause substrate/chemical compound precipitate. (Important!).

A protocol for 50µl reaction containing 80nM GST-3CLPro and 20µM Substrate

Experimental Design:

Component:	**Compound/Buffer**	**GST-3CLPro**	**Dabcyl-KTSAVLQSGFRKME-Edans**
Volume:	30µl	10µl	10µl
Concentration:	Up to your assay	80nM	20µM

A. Prepare assay components

A.1 Dilute the provided 5X Assay buffer using ice-cold deionized water to make 1X Assay buffer. Make enough 1X Assay buffer to dilute kit components and chemical compounds.

A.2 Chemical compounds are often prepared in an organic vehicle (such as DMSO). Prepare necessary amount of 1X Assay buffer with vehicle (such as 1-2% DMSO based on your compound dilution, limit DMSO no more than 2% in the final reaction). This buffer is defined as 1X Assay buffer with vehicle.

A.3 Dilute appropriate amount of 10X GST-3CLPro using cold 1X Assay buffer prepared in STEP A.1. Pipet to mix well. The diluted GST-3CLPro concentration is 400nM. Keep the diluted Enzyme on ice first and move it to room temperature once other components are ready.

A.4 Move the remaining 1X Assay buffer to room temperature and wait for 20min to warm.

A.5 In a centrifuge tube, transfer appropriate amount of 100X Dabcyl-KTSAVLQSGFRKME-Edans to the bottom of the tube, then add the necessary amount of room temperature 1X Assay buffer in once and immediately pipet to mix well (see Note 5 above). The diluted Substrate concentration is 100µM.

A.6 In a centrifuge tube, transfer appropriate amount of 100X PF-07321332 (a 3CLPro inhibitor) to the bottom of the tube, then add the necessary amount of room temperature 1X Assay buffer in once and immediately pipet to mix. This diluted Inhibitor is 33.4µM.

A.7 Use the same procedure in STEP A.6 to prepare your chemical compounds if performing a compound screening or profiling assay. Limit DMSO no more than 2% in the final reaction.

B. Set up reactions

B.1 Include the following reactions in an assay:

- Positive control contains GST-3CLPro (Enzyme) and Dabcyl-KTSAVLQSGFRKME-Edans (Substrate)
- Substrate control contains Dabcyl-KTSAVLQSGFRKME-Edans only
- Positive Inhibition control contains PF-07321332, GST-3CLPro, and the Substrate
- Test compound control contains your compound only (to test if your compound has autofluorescence under the assay condition)
- Test compound assay contains a test compound, GST-3CLPro, and the Substrate
- Triplicates are recommended for each reaction

B.2 In a 96-well clear flat bottom black microplate, add 10µl diluted GST-3CLPro enzyme prepared in STEP A.3. For control reactions containing the substrate only (Dabcyl-KTSAVLQSGFRKME-Edans) or a test compound only, add 10µl 1X Assay buffer with vehicle prepared in STEP A.2 to replace the enzyme.
Note: you can use non-clear bottom microplate if your plate reader can read from the top.

B.3 Add 30µl PF-07321332 prepared in STEP A.6 or your chemical compound diluent prepared in STEP A.7 to corresponding wells. The final PF-07321332 concentration in the reaction is 20µM after the substrate is added as well. For control reaction wells that do not have any chemical compound, add 30µl 1X Assay buffer with vehicle. Gently mix the solutions using a microplate shaker for 1min. Incubate under room temperature for 30 min to allow potential compound/enzyme interactions. Protect the plate from light if necessary.
Note: We did a 2X series of dilution of PF-07311332 to set up reactions (*Figure B below*).

C. Measure the enzymatic reaction

Note: The plate reader setting should be optimized using the kit, and the plate reader is ready-to-use before you set up the assays.

C.1 Add the substrate to initiate reactions

- For control reactions without the substrate, add 10µl 1X Assay buffer into corresponding wells.
- For reactions with the substrate, add 10µl diluted Dabcyl-KTSAVLQSGFRKME-Edans (Substrate) prepared in STEP A.5 to corresponding wells. This final Substrate concentration in the reaction is 20 µM. Gently shake the plate for 1-2min to mix the solutions using a microplate shaker (or use your plate reader to mix the solutions if available). Transfer the plate to your ready-to-use plate reader immediately.

C.2 Measure fluorescence

Note: The reaction time in both kinetic and endpoint modes could be shorter or longer than 60min depending on your plate reading settings and sensitivity.

- For kinetic reading, immediately measure the fluorescence intensity at Ex/Em = 360/530nm continuously and record data every 2-3 min for a period of 60 min. Gently shake the plate before each reading if your plater reader has a shaking function.

- For endpoint reading, once the substrate is added, gently mix the solution for 1-2min using a microplate shaker, keep the plate from light, and incubate under room temperature for 60 min. After incubation, gently shake the plate for 1-2min and measure the fluorescence intensity at Ex/Em = 360/530nm.

D. Data analysis

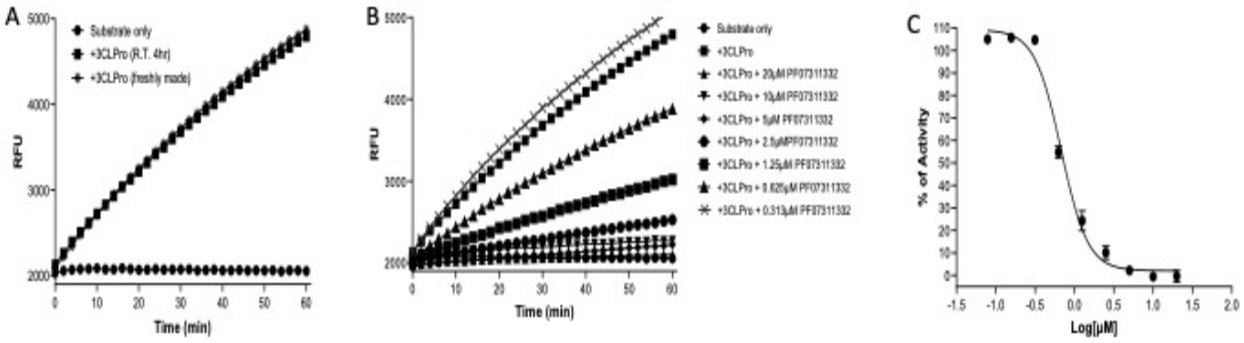
D.1. For kinetic assay

- Determine the time range during which the fluorescence intensity increases linearly.
- Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of each reaction. For example, if all reactions have a linear fluorescence increase in the first 10 min of reaction, then $V_0 = (Y_{10} - Y_0)/10$. Y_{10} is the RFU value at 10min in Y axis, Y_0 is the RFU value at 0min in Y axis.
- V_o from the substrate control should be subtracted as the background.
- If a compound has autofluorescence, subtract the compound alone V_o as the background for corresponding reactions containing the compound.
- The V_o value after background correction represents the enzyme activity in each well.
- The V_0 value in reactions containing 3CLPro and the Substrate (without an inhibitor) can be referenced as 100% activity. Percentage of remaining 3CLPro activity in reactions containing an inhibitor can be calculated by dividing the corresponding V_0 value with the V_0 value without an inhibitor.
- Plot percent of activity of a testing compound (Y axis) vs. Log[concentration] of compound (*Figure C below*) can be done to determine inhibition percentage, EC_{50}, IC_{50}, etc.

D.2. For endpoint assay

- RFU from the substrate only and/or compound only should be subtracted as the background.
- The absolute RFU value after background correction represents the 3CLPro activity in each well.
- The background corrected RFU value in reactions containing 3CLPro and the Substrate (without an inhibitor) can be referenced as 100% activity. Percentage of 3CLPro activity in reactions with an inhibitor can be calculated by dividing the corresponding V_0 value with the V_0 value without an inhibitor.
- Plot percent of 3CLPro activity (Y axis) vs. Log[concentration] of a compound can be used to determine inhibition percentage, EC_{50}, IC_{50}, etc.

E. Results



A. Reactions contained 20μM Dabcyl-KTSAVLQSGFRKME-Edans and 80nM GST-3CLPro. 4μM GST-3CLPro stock was prepared right before the reaction or kept under room temperature for 4 hours prior to addition to reactions.
B. Reactions contained 20μM Dabcyl-KTSAVLQSGFRKME-Edans and 80nM GST-3CLPro in the presence of 2% DMSO, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125μM PF-07321332.
C. GST-3CLPro activity was measured as shown in B. % of GST-3CLPro activity was plotted vs. Log[μM] of PF-07321332. Data were shown as mean ± SD of triplicates.