UBPBio
Ubiquitin-Proteasome Biotechnologies

PLPro Deubiquitinase Activity Assay Kit

Kit I – Cat# J7650 (120 assays)
Kit II – Cat# J7651 (1,200 assays)

The kit measures the deubiquitinase activity of SARS-CoV-2 papain-like protease (PLPro), can be used for screening/profiling PLPro inhibitors. Kit I is sufficient for 120 X 50µl reactions; Kit II is sufficient for 1,200 X 50µl reactioons. Optimized for rapidly and quantitatively measuring PLPro activity under room temperature.

For detecting PLPro's proteinase activity, refer to the PLPro Proteinase Activity Assay Kit (cat# J7610 or J7611) that uses Dabcyl–FRLKGGAPIKGV–Edans as the fluorogenic substrate.

Key Features:

- Optimized for room temperature operation, particularly useful for HTS assays
- Rapid and quantitative result within 2-3 hours
- Positive PLPro inhibitor GRL-0617 included
- Large excess amount of buffer provided
- High concentration component stocks allowing custom assay development

Component Information

Components	Quantity (Kit I, J7650)	Quantity (Kit II, J7651)
10X PLPro (Enzyme)	3µM, 120µl	3µM, 1.2ml
10X Ubiquitin-Rhodamine 110 (Substrate)	37.5µM, 120µl	37.5µM, 1.2ml
100X GRL-0617 (Inhibitor)	10mM in DMSO, 10µl	10mM in DMSO, 20µl
5X Assay Buffer	2 X 1.2ml	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 485/530 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 PLPro and the fluorogenic substrate in 1X Assay Buffer are stable for at least 4 hours under room temperature.
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 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 chemical compounds. Do not reverse the order by adding a chemical compound stock into 1X Assay buffer as this may cause the chemical compound precipitate. (Important!).

A protocol for 50µl reaction containing 60nM PLPro and 0.75µM Substrate

Experimental Design:

Component:	**Compound/Buffer**	**PLPro**	**Ubiquitin-Rhodamine 110**
Volume:	30µl	10µl	10µl
Concentration:	Up to your assay	60nM	0.75µM

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 PLPro using 1X cold Assay buffer prepared in STEP A.1. Pipet to mix well. The diluted PLPro concentration is 300nM. 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 20 min to warm.

A.5 In a centrifuge tube, transfer appropriate amount of 10X Ubiquitin-Rhodamine110 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. The diluted Substrate concentration is 3.75µM.

A.6 In a centrifuge tube, transfer appropriate amount of 100X GRL-0617 (a PLPro 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. The diluted Inhibitor is 100µM.

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 PLPro (Enzyme) and Ubiquitin-Rhodamine 110 (Substrate)

- Substrate control contains Ubiquitin-Rhodamine 110 only
- Positive Inhibition control contains GRL-0617, PLPro, 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, PLPro, and the Substrate
- Triplicates are recommended for each reaction

B.2 In a 96-well clear flat bottom black microplate, add 10µl diluted PLPro enzyme prepared in STEP A.3. For control reactions containing the substrate only (Ubiquitin-Rhodamine 110) 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 GRL-0617 prepared in STEP A.6 or your chemical compound diluent prepared in STEP A.7 to corresponding wells. The final GRL-0617 concentration in the reaction is 60µM 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 1-2min. Incubate under room temperature for 30min to allow potential compound/enzyme interactions. Protect the plate from light if necessary.
Note: We did a 2X series of dilution of GRL-0617 to set up reactions (See *Figure 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 Ubiquitin-Rhodamine 110 (Substrate) prepared in STEP A.5 to corresponding wells. The final Substrate concentration in the reaction is 0.75µM. 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 = 485/530nm 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 = 485/530nm.

D. Data analysis

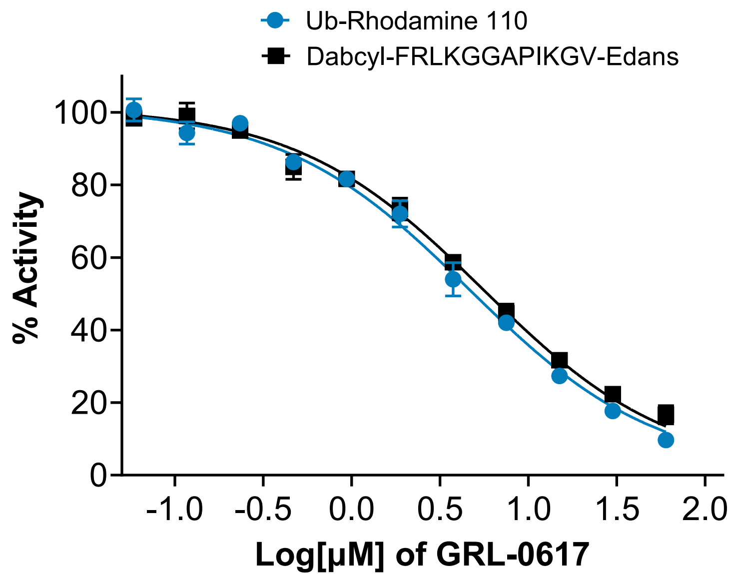
D.1. For kinetic assay

- 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 PLPro and the Substrate (without an inhibitor) can be referenced as 100% activity. Percentage of remaining PLPro 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 (See *Figure 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 PLPro activity in each well.
- The background corrected RFU value in reactions containing PLPro and the Substrate (without an inhibitor) can be referenced as 100% activity. Percentage of PLPro 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 PLPro activity (Y axis) vs. Log[concentration] of a compound can be used to determine inhibition percentage, EC_{50}, IC_{50}, etc.

E. Results



Inhibition of PLPro's proteinase or DUB activity by GRL-0617. Reactions contained 20µM Dabcyl–FRLKGGAPIKGV–Edans and 150nM PLPro (Squire symbols), or 0.75 Ub-Rhodamine110 and 60nM PLPro (circle symbols). GRL-0617 was added as a series of 2X titration in reactions. PLPro activity without GRL-0617 was referenced as 100% activity. % of PLPro activity was plotted vs. Log[µM] of GRL-0617. Data were shown as mean ± SD of triplicates.