UbiQ
targeting the ubiquitin system

UbiQ protocol P009 _ Characterization of SARS-CoV-1 and SARS-CoV-2 Papain-Like Protease
ISG15 and Ub based reagents

Description: Characterization of the Ubiquitin and ISG15 Deconjugation Activity of SARS-CoV-1 and SARS-CoV-2 Papain-Like Protease (PLpro)
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Abstract

Both severe acute respiratory syndrome coronavirus 1 and 2 (SARS-CoV-1 and SARS-CoV-2) encode a papain-like protease (PLpro), which plays a vital role in viral propagation. PLpro accomplishes this function by processing the viral polyproteins essential for viral replication and removing the small proteins, ubiquitin and ISG15 from the host's key immune signaling proteins, thereby preventing the host's innate immune response. Although PLpro from both SARS-CoV-1 and SARS-CoV-2 are structurally highly similar (83% sequence identity), they exhibit functional variability. Hence, to further elucidate the mechanism and aid in drug discovery efforts, the biochemical and kinetic characterization of PLpro is needed. This chapter describes step-by-step experimental procedures for evaluating PLpro activity in vitro using activity-based probes (ABPs) along with fluorescence-based substrates. Herein we describe a step-by-step experimental procedure to assess the activity of PLpro in vitro using a suite of activity-based probes (ABPs) and fluorescent substrates and how they can be applied as fast and yet sensitive methods to calculate kinetic parameters.

1. Introduction.

After the discovery of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1, 2] around 2019 December, the virus has already spread across all the continents of the world with more than 100 million confirmed cases and more than five million fatalities till date [3]. SARS-CoV-2 is the causative agent for novel coronavirus disease 2019 (COVID-19) with influenza-like symptoms including fever or chills, cough, headache, diarrhoea, etc., with the most prominent indicators being shortness of breath and loss of taste or smell. The emergence of COVID-19 as a global pandemic serves as the third example of causing acute disease in humans, with SARS-CoV-1 outbreak being the first, beginning in 2002, followed by the appearance of the Middle East respiratory syndrome (MERS-CoV) around 2012 [4]. Although SARS-CoV-2 shares high sequence similarity and disease physiology with SARS-CoV-1, SARS-CoV-2 has fewer case fatality and increased infectivity, which leads to quick and worldwide transmission [5, 6]. While there have been recent developments with emergency approvals of vaccines, which have reduced the spread, there have been cases of new variants which may bypass the immune responses developed by vaccines. Hence there is a dire need to develop antiviral drugs with enhanced efficacies to combat COVID-19.

Both SARS-CoV-1 and SARS-CoV-2 encode for a papain-like cysteine protease (PLpro) [7], which has been a significant focus for drug development. In the host, this enzyme promotes viral replication by processing the viral encoded single-chain nonstructural proteins (NSPs) into individual subunits via proteolytic hydrolysis of the peptide bonds [8]. These individual proteins (termed NSP1, NSP2, etc.) help in viral genome replication, among other functions [9]. It was also later identified that PLpro has deubiquitinating and deISGylating activity. Here, PLpro cleaves the isopeptide bond between host cellular proteins and the small proteins ubiquitin (Ub) and ISG15 (Interferon Stimulating gene 15), respectively [10–12]. Since posttranslational modifications with Ub and ISG15 play a crucial role in the innate immune response to viral infection, PLpro-induced de-conjugation of these modifiers promotes viral pathogenesis. Moreover, PLpro is also responsible for inhibiting key innate immune molecules like cytokines and chemokines [13–15]. Hence, it is no surprise that PLpro is one of the key targets for therapeutic intervention.

Various structures for PLpro from both SARS-CoV-1 and recently from SARS-CoV-2 have been reported, in both apo form and in complex with suitable substrates. Also, many structural studies have identified small molecules that impede PLpro function [7, 16, 17]. However, prerequisite to structural studies pertaining to small molecules, it is imperative to set up a platform to assess the activity of PLpro in vitro for high-throughput studies. Also, it was vital for PLpro/substrate structures to stabilize the complex because of the low affinity between them. Although the PLpro structures provided a mechanistic understanding of the activity of this enzyme, they did not shed light on their kinetics with respect to various substrates (like Ub versus ISG15). However, with technical advances, these substrates were modified, which could be used to capture or measure the activities of PLpro and other deubiquitinating/deISGylating enzymes [18–20]. One of the modifications included the generation of activity-based probes (ABPs) [21].

ABPs are substrate mimetics that present an electrophilic "warhead" to the active site nucleophile to produce a stable, covalent adduct. Application of these ABPs was indispensable in capturing the PLpro/substrate complex for structural studies [22–25]. In this chapter we will focus on ABPs equipped with the vinyl pentynyl sulfone (VPS) warhead, an electrophile that is also suitable for further functionalization by click chemistry [26]. The described ABP protocol is also applicable to Ub and ISG15 ABPs equipped with other electrophiles [23]. Despite all the merits of these ABPs, they are inadequate when it comes to kinetic information, which is important in understanding substrate specificity. Various (quenched) fluorescent substrates are commercially available. The most known substrates are equipped with 7-amido-4-methylcoumarin (AMC) or Rhodamine110Gly (Fig. 1). Although AMC is very robust and easy to use, its relatively low excitation and emission spectrum (exc 380/emi 460 nm) may coincide with many small molecules, and hence the Rh110Gly substrates are preferred (exc 485/emi 520 nm). A recent improvement of the Rh110Gly substrates is based on the introduction of Rh110-morpholinecarbonyl (Rh110MP) [27], which has a similar excitation and emission spectrum as Rh110Gly but exhibits a much higher fluorescence intensity.

Since both AMC- and Rh110-based substrates do not represent true substrate mimetics due to the absence of the native isopeptide linkage, a TAMRA-K(Ub)G and TAMRA-K(ISG15)G substrate were developed [28] (Fig. 1). Here Ub or ISG15 is linked via an isopeptide bond to a tetramethylrhodamine (TAMRA) labeled Lys-Gly dipeptide. Protease activity is measured through fluorescence polarimetry (FP). This chapter describes step-by-step instructions on how to use the previously described suite of reagents to profile PLpro activity with necessary recommendations such that one can get reproducible results.

2 Materials

2.1 Protein Preparation

1. Plasmids expressing glutathione S-transferase (GST)-tagged versions of SARS-CoV-1 and SARS-CoV-2 PLpro proteins with codon usage optimized for expression in Escherichia coli. The plasmids carry the synthetic genes in the pGEX6P-1 vector (GE Healthcare, UK) at the BamHI and NotI sites and are synthesized by Gene Universal (USA) for expression as a Pre-Scission protease-cleavable N-terminally GST-tagged protein28.
2. Bacterial strain E. coli BL21(DE3) RIL Codon Plus (Stratagene).
3. 100-mg ampicillin per mL in water, filter sterilized.
4. 1-M isopropyl-b-d-thiogalactopyranoside (IPTG) in water, filter sterilized.
5. 1-M ZnSO4 in water, filter sterilized.
6. LB broth.
7. Lysis buffer: 50 mM Tris–Cl pH 8.0, 300 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM β-mercaptoethanol.
8. Equilibration buffer: 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP).
9. Refrigerated shaker (Innova 44R; New Brunswick Scientific).
10. Sonicator (Branson 450 Digital Sonifier).
11. Glutathione Sepharose 4B affinity chromatography resin (GE Healthcare).
12. PreScission protease (Cytiva).
13. A stock solution of 0.5-M reduced glutathione, pH 8.0 in water.
14. Chromatography systems: NGC purification system (Bio-Rad), equipped with gel filtration (Superdex-200 pg 26/60) and desalting (HiPrep 26/10) columns (GE Healthcare).
15. Glass Econo-Columns, 2.5 × 20 cm (Bio-Rad).
16. Centrifugal filter units: Amicon Ultra-4 and Ultra-15 (10-kDa molecular weight cut-off, MWCO) (Millipore).
17. Liquid nitrogen.

2.2 ABPs and Gel-Based Assay Preparation

1. Ub-VPS (UbiQ Bio B.V., product code UbiQ-193).
2. Ac-mISG15-VPS (C-terminal domain of mouse ISG15) (UbiQ-262).
3. Ac-hISG15-VPS (C-terminal domain of human ISG15) (UbiQ-311).
4. 100% DMSO.
5. 2.5-M sodium acetate buffer, pH 4.5.
6. 1-M HEPES buffer, pH 7.5.
7. 10× assay buffer: 500 mM HEPES pH 7.5, 1500 mM NaCl, 5 mM TCEP.
8. 4× SDS reducing loading buffer.
9. 4–20% gradient gels (Bio-Rad) or homemade gels.
10. Coomassie Brilliant blue stain (Bio-Rad, 1610436).
11. Standard electrophoretic equipment.

2.3 Fluorescent Substrates and Plate- Based Assay Preparation

1. Ub-AMC (R&D systems, Catalog# U-550-050).
2. Ub-Rh110MP (UbiQ Bio B.V., product code UbiQ-126).
3. TAMRA-Lys(Ub)-Gly (UbiQ Bio B.V., product code UbiQ- 012).
4. ISG15-AMC (R&D systems, Catalog# U-553-050).
5. Ac-mISG15-Rh110MP (C-terminal domain of mouse ISG15) (UbiQ Bio B.V., product code UbiQ-127).
6. Ac-hISG15-Rh110MP (C-terminal domain of human ISG15) (UbiQ Bio B.V., product code UbiQ-309).
7. 100% DMSO.
8. Black 384-well assay plate, flat bottom, low flange (Corning, REF 3575).
9. PHERAstar plate reader equipped with suitable optic modules (BMG Labtech).
10. 10× assay buffer: 500 mM HEPES pH 7.5, 1500 mM NaCl, 5 mM TCEP.
11. Multichannel pipette.

2.4 Data Analysis

1. MARS software (version 3.20 R2).
2. Microsoft Excel (version 14.0.0 (100825)).
3. GraphPad (version 7.0a).

3 Methods

3.1 Preparing the PLpro for the Assay

1. Transform the pGEX6P-1-based plasmids designed to express the GST-tagged versions of SARS-CoV-1 and CoV-2 PLpro into E. coli strain BL21 (DE3) Codon Plus.
2. Grow the cells in LB broth supplemented with 100-µg/mL ampicillin at 37 °C with shaking until the optical density at 600 nm reaches 1.5.
3. Add 0.2 mM isopropyl-β-D-thiogalactopyranoside and 0.1- mM $ZnSO_4$ to induce protein expression followed by shaking overnight at 18 °C.
4. Harvest cells by centrifugation (6000 g for 15 min at 4°). Resuspend the cell pellets in lysis buffer.
5. In a beaker placed on an ice water bath, disrupt the cell pellets by sonication (5 min with 50% amplitude for 2 s on and 5 s off). Clear the lysate by centrifugation at 35,000 g for 30 min at 4 °C.
6. Meanwhile, pour 5–10 mL of Glutathione Sepharose 4B resin into the Econo-Column, and wash the beads with MilliQ water to remove the ethanol solution from the beads. Again, wash the beads with the lysis buffer to equilibrate them.
7. Load the cleared lysis supernatant onto Glutathione Sepharose 4B resin, placed in the Econo-Column, and allow the supernatant to pass slowly. Wash the proteins bound to the resin with lysis buffer.
8. Elute the GST-tagged proteins in lysis buffer supplemented with 30 mM reduced glutathione pH 8.0.
9. Concentrate the fusion proteins in Amicon Ultra-15 centrifugal filters (10 kDa, MWCO) until 10 mL.
10. Add GST–PreScission protease at 1:1000 (w/w) to the protein samples and incubate overnight at 4°C to cleave the GST tag.
11. Inject 10 mL of the target protein with cleaved GST tag and PreScission protease into a desalting column (HiPrep 26/10 desalting) to remove the glutathione.
12. Passage the fractions collected from the desalting column through fresh Glutathione Sepharose 4B resin to remove the cleaved GST and PreScission protease.
13. Concentrate the cleaved proteins in centrifugal filters 10 kDa (Amicon Ultra-15) until 10 mL, and further purify using Superdex-200 pg 26/60 size-exclusion columns with equilibration buffer.
14. After purification, concentrate the proteins to 5–10 mg/mL, using Amicon Ultra-15 or Ultra-4, aliquot for single use, and snap freeze in liquid nitrogen (see Note 1).

3.2 Gel-Based Assay Using Activity-Based Probes (ABPs)

3.2.1 Refolding and Stock Solution Preparation for the ABPs

1. All UbiQ probes are supplied as 50-µg lyophilized powder in tubes.
2. Dissolve the CTD mISG15-VPS and CTD hISG15-VPS probes in 2-µL DMSO. Add this solution to 91-µL MilliQ water followed by the addition of 2 µL of 2.5-M sodium acetate pH 4.5 (final concentration of 50 mM sodium acetate). Buffer these stock solutions with assay buffer by adding 5 µL of 1-M HEPES pH 7.5, resulting in final probe concentrations of 52 µM (0.5 mg/mL) and 54 µM (0.5 mg/mL), respectively, and a final pH of pH 7.5 (see Note 2).
3. Dissolve the Ub-VPS probe in 2-µL DMSO and add this stock to 93-µL MilliQ water followed by the addition of 5 µL of 1-M HEPES pH 7.5 to generate a final probe concentration of 50 µM (0.5 mg/mL) (see Note 3).

3.2.2 Setting Up the Assay

1. Perform the entire experiment at 4 °C (see Note 4).
2. Dilute all protein samples in 1× assay buffer and incubate the CTD mISG15-VPS probe stock (prepared in previous section) and proteins at 4 °C before starting the experiment.
3. Spin both the probe and PLpros at high speed on a micro- centrifuge to remove any precipitate.
4. Keep the final reaction volume at 120 µL in 1× assay buffer. Maintain the PLpro and probe concentration at 1 µM and 3 µM, respectively (see Note 5). Add the PLpro first and start the reaction by adding the CTD mISG15-VPS probe stock (see Note 6).
5. Collect samples (15 µL) at 2, 5, 10, 30, 60, and 120 s, respectively (see Note 7), and add to 1.5-mL micro-centrifuge tubes pre-dispensed with 5-µL 4× SDS reducing loading buffer to quench the reaction.
6. Prepare a negative control by incubating a sample with only 1-µM PLpro for 120 s.
7. Spin the above loading dye-treated samples, including negative control, and load 5–7 uL of the supernatant on a 4–20% gradient gel. Stain the gel using Coomassie blue (see Note 8) (Fig. 2).
8. Repeat the above steps (1–6) for CTD hISG15-VPS and Ub-VS probes, respectively.

3.3 Kinetic Assay Using Fluorescent Substrates

3.3.1 Preparation of Stock Solutions of Fluorescent Substrates

1. Reconstitute UbiQ-126, UbiQ-127, UbiQ-309, and UbiQ-012 as discussed before for ABPs: dissolve the reagent in DMSO, add to MilliQ water, and buffer with 50 mM sodium acetate pH 4.5. Finally, buffer this stock using 1-M HEPES pH 7.5, affording a final stock of 50 µM (see Notes 2 and 3).
2. Dilute UbiQ-126 and UbiQ-012 to 20-µM stocks, and dilute UbiQ-127 and UbiQ-309 to 10 µM in 1× assay buffer (2× working stock).
3. Directly dilute Ub-AMC and ISG15-AMC (R&D systems) in 1× assay buffer to a final concentration of 20 µM and 10 µM (2× working stocks), respectively.

3.3.2 Preparation of Kinetic Assay with the Fluorescent Probes

1. Perform all kinetic assays at room temperature, i.e., 25 °C (see Note 9).
2. Perform the assay in a black 384-well format assay plate (see Note 10), keeping the final reaction volume at 20 µL.
3. Dilute PLpro to 40 nM (2× working stocks) in 1× assay buffer.
4. In the first well of the assay plate, add 20 µL of 2× working stock of fluorescent probe (Ub-AMC). Using a multichannel pipette, add 10 µL of 1× assay buffer to the subsequent seven wells. Then using a single-channel pipette, transfer 10 µL of the probe from the first well to the second well and mix properly. Next, transfer 10 µL of the probe from the second well to the third well and mix. Repeat this procedure until the eighth well, such that each well has 10 µL of solution. Hence the substrate is serially diluted twofold to achieve eight different concentrations (see Note 11).
5. Prepare two 20-µL samples as negative controls, including 1× working stock of PLpro (well 9) and the probe (well 10), respectively.
6. Place the plate into the PHERAstar plate reader and initiate the reaction by adding 10 µL of 2× working stock of PLpro to wells 1–8. Start the data collection immediately in kinetic plate mode (described in Subheading 3.3.6).
7. Repeat the above protocol two to five times to get statistically relevant kinetic data.
8. Repeat the above steps (1–6) to generate data for Ub-Rh110MP, ISG15-AMC, CTD mISG15-Rh110MP, and CTD hISG15-Rh110MP, respectively.

3.3.3 Preparation of the Standard Curve

1. Serially dilute the fluorescent substrate probes (except UbiQ-012) twofold maintaining the same concentrations described above with a reaction volume of 20 µL (see Note 12).
2. Place the plate in the PHERAstar plate reader and obtain a baseline reading under the endpoint mode (described in Sub- heading 3.3.5). This measurement serves as the background fluorescence from the probes.
3. Add a minimal volume (preferably 0.5–1 µL) of concentrated PLpro (CoV-1-PLpro) to those eight wells. After 5 min of incubation, take a reading in the plate mode (see Note 13).
4. Continue measuring the reads every 5–7 min until the signal stops increasing further.
5. Subtract the last fluorescence reading in step 4 from step 2 and save it on an Excel sheet.
6. Repeat the above steps for all the substrate fluorophores (except UbiQ-012). Next, we describe step-by-step instructions on preparing the plate reader to measure endpoint and plate mode readings for standard curve and enzyme kinetics, respectively.

3.3.4 Setting Up the PHERAstar Plate Reader

1. After opening the PHERAstar application, look for the "manage protocol" tab and click on the "New" tab.
2. Under "measurement method," choose "Fluorescence Intensity" for UbiQ-126, UbiQ-127, UbiQ-309, Ub-AMC, and ISG15-AMC or "Fluorescence Polarimetry" for UbiQ-012.
3. Next, under "reading mode," choose either "Endpoint" for making the standard curve or "Plate mode" for kinetic analysis. When "Endpoint" mode is executed, only one read is collected. But in the "Plate mode," continuous reads are collected depending on the "no. of cycles" entered (explained in Subheading 3.3.6).

3.3.5 Using Endpoint Mode (for Generating Standard Curve)

1. Under the Basic parameter tab, for the "Microplate" drop- down menu, select Corning 384-Black low flange (see Note 14).
2. Next, choose the correct optic module based on the probe used. For AMC, Rhodamine110, and TAMRA, select FI 355460, FI 485520, and FP 540-20 590-20, respectively (see Note 15).
3. Choose "top" for the optic option. Keep all other settings as default.
4. Under the layout tab, choose the wells for which data needs to be collected. Here use the option to label certain wells as a positive or negative control.
5. Click on "Start measurement."
6. On the new page, select "optical focal and gain adjustment." For gain adjustment, set the target value as 60–80% and click "start adjustment" (see Note 16).
7. Click "measure one plate" to take the reading (see Note 17).

3.3.6 Using Kinetic Plate Mode (for Kinetic Assay)

1. The parameters that appear under this option remain almost the same as endpoint mode. Thus, choose similar options as discussed above. There is an additional window called "Kinetic Window 1," which gives the option for entering "no. of cycles" and "cycle time."
2. Enter 1000 for a number of cycles and 9 s as cycle time (see Note 18).
3. As discussed before, under the "layout window," choose the samples for which the data will be collected and click "start measurement."
4. On the new page, choose appropriate values of focal height and gain adjustment (see Note 19).
5. Click "Measure one plate" to collect data (see Note 17)

3.4 Data Analysis

3.4.1 Generation of Standard Grap

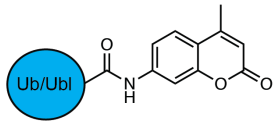
1. Open the Excel file generated in Subheading 3.3.3.
2. Plot a curve using the chart option keeping the concentration (µM) on X-axis and fluorescence reading on Y-axis.
3. Under the "format trendline" tab, select "display equation" and "R-squared value" on the chart (see Note 20).
4. Similarly, generate equations and R-squared values for all the probes.

3.4.2 Analysis of the Raw Kinetic Data

1. Copy the data generated in the kinetic mode from the PHERAstar page and paste it into the Excel sheet. These data include the fluorescence reading and time intervals for each substrate concentration used.
2. Normalize the data such that at time point = 0, there was no fluorescence for each substrate concentration (see Note 21).
3. Then using the equations generated in the "Standard curve" section above, convert the fluorescence reading to concentration (µM) for each substrate concentration.
4. Based on the data generated in step 3, plot a graph using Concentration on the Y-axis and Time on the X-axis.
5. Calculate the slopes for each substrate concentration from step 4 and save this information on an Excel sheet. These values serve as the rate of reactions or enzyme activities for each fluorophore substrate (see Note 22).

3.4.3 Generation of Kinetic Parameters

1. Open GraphPad Prism and under "Use tutorial data," choose the last option for more tutorials.
2. Under the drop-down menu, choose "Michaelis-Menten" equation and click "Create."
3. On the new page, remove the prefilled data under "Substrate" and "Enzyme activity" tab; enter the values for the used fluorescent substrate concentration (Subheading 3.3.2) and rate of reaction generated from the previous section ("Polishing Raw kinetic data").
4. Click on "Analyze" and choose Non-linear regression (curve fit).
5. In the next page, choose "Michaelis-Menten" for fitting the data. By default, the fitting method used is least squares (ordinary) fit.
6. Calculate the kinetic parameters like V_{max} and K_m, including the Michaelis–Menten graph. Calculate K_{cat} based on the standard formula: $K_{cat} = V_{max}/E$ where E is the PLpro concentration (Figs. 3 and 4) (see Note 23).

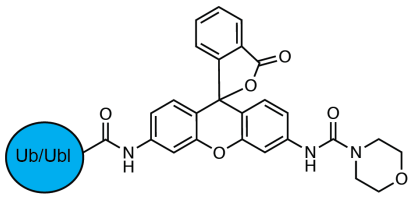


AMC

excitation / emission: 380 nm / 460 nm

Pros:
1: easy to use and amenable for high-throughput assay.

Cons:
1: does not mimic true substrate.
2: excitation wavelength is in the UV range, hence it is not optimal for drug discovery as it can excite many screening compounds and cause false positives

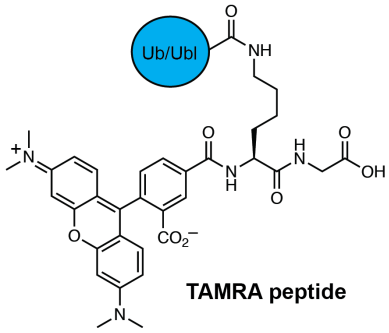


Rh110MP

excitation / emission: 485 nm / 520 nm

Pros:
1: easy to use and amenable for high-throughput assay.
2: excitation wavelength is outside UV range, making it very suitable for screening small molecules.

Cons:
1: does not mimic true substrate.



TAMRA peptide

excitation / emission: 540 nm / 590 nm

Pros:
1: easy to use and amenable for high-throughput assay.
2: excitation wavelength is outside UV range, making it very suitable for screening small molecules.
3: mimics a true substrate as Ub/Ubl is linked via an isopeptide linkage

Figure 1. Fluorophore substrates used in this chapter. The chemical structures of fluorophores with their excitation/emission wavelengths and other properties are shown. AMC= 7-amido-4-methylcoumarin, Rh110MP= Rhodamine110-morpholinecarbonyl, TAMRA= tetramethylrhodamine.

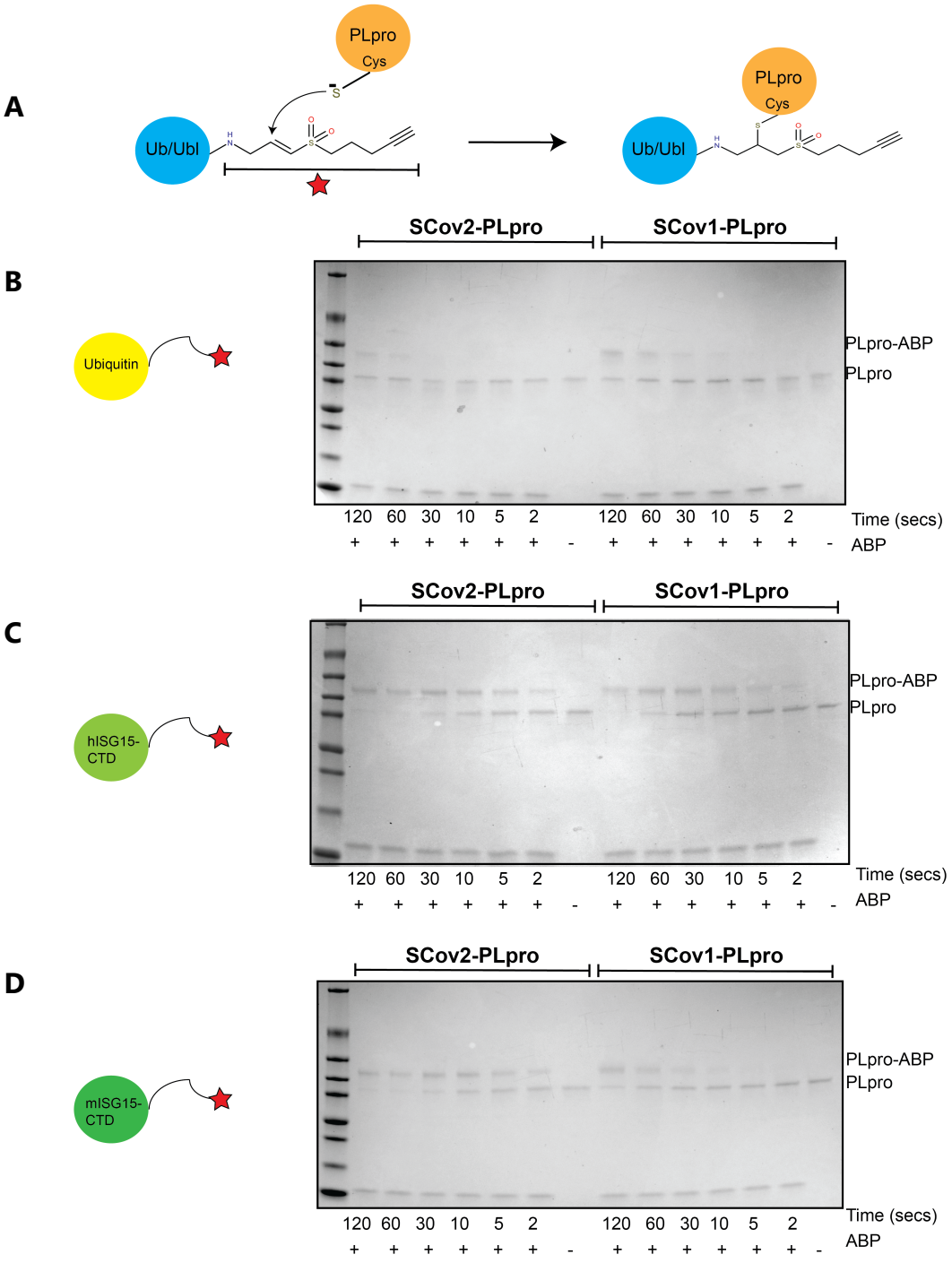


Figure 2. Summary of results for gel-based assays using activity-based probes (ABPs). (a) Chemical structure of vinyl pentynyl sulfone (VPS) and its mode of action with the PLpro active site cysteine. (b) Gel-based assay to show qualitative labeling of PLpro from SARS-CoV-1 (left part of gel) and SARS-CoV-2 (right part of gel) using Ub-VPS. Similar labeling uses the C-terminal domain of human ISG15-VPS (c) and the C-terminal domain of murine ISG15-VPS (d).

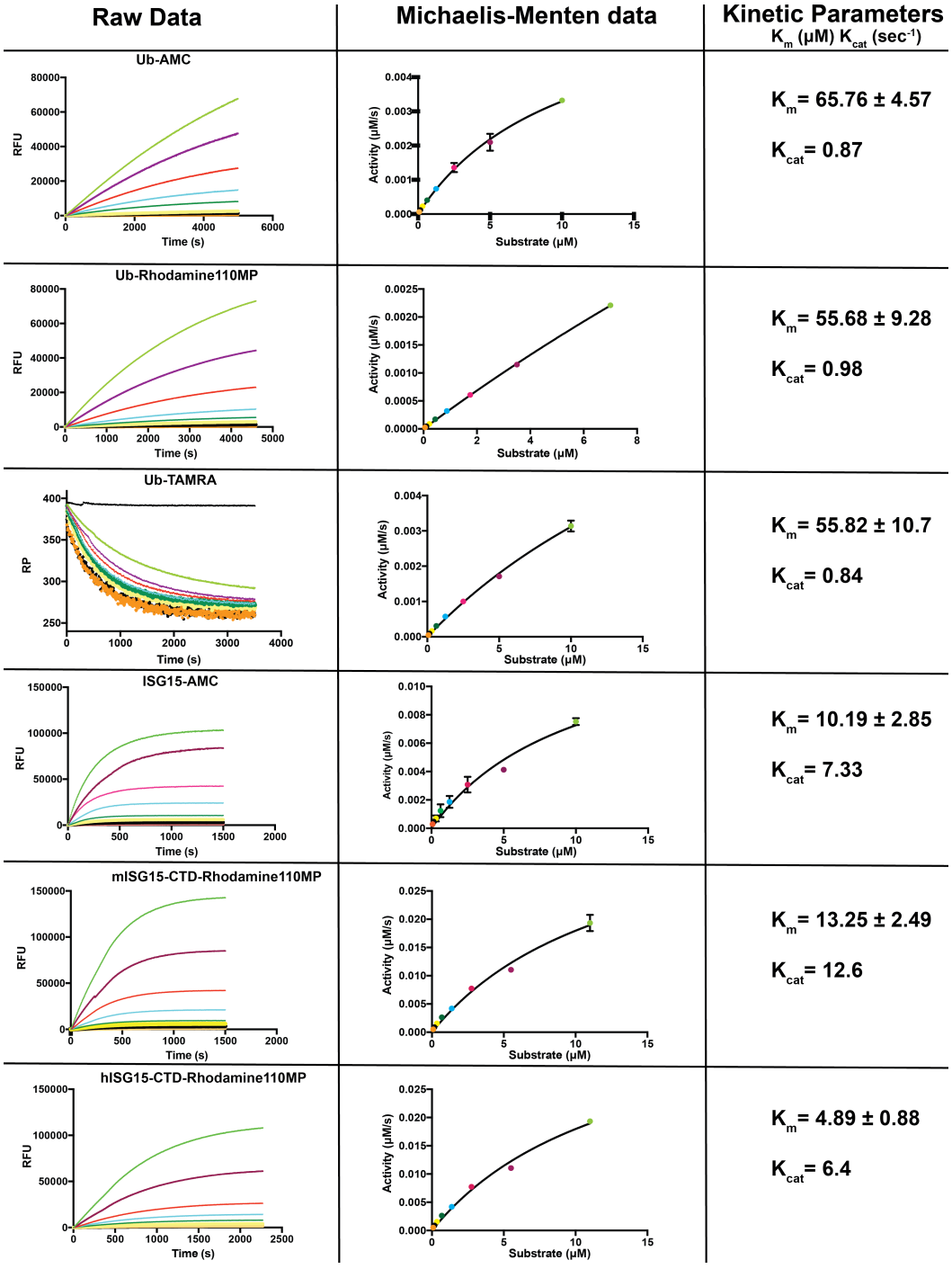


Figure 3. Summary of results showing Michaelis–Menten kinetic parameters for SARS-CoV-1 PLpro using various fluorescently labeled substrates. Shown here is the plot between relative fluorescence unit (RFU) and relative polarimetry (RP) with time in seconds, termed as "raw data." Each color represents substrate concentration. Using the raw data, the Michaelis–Menten graph was plotted to calculate kinetic parameters using GraphPad software.

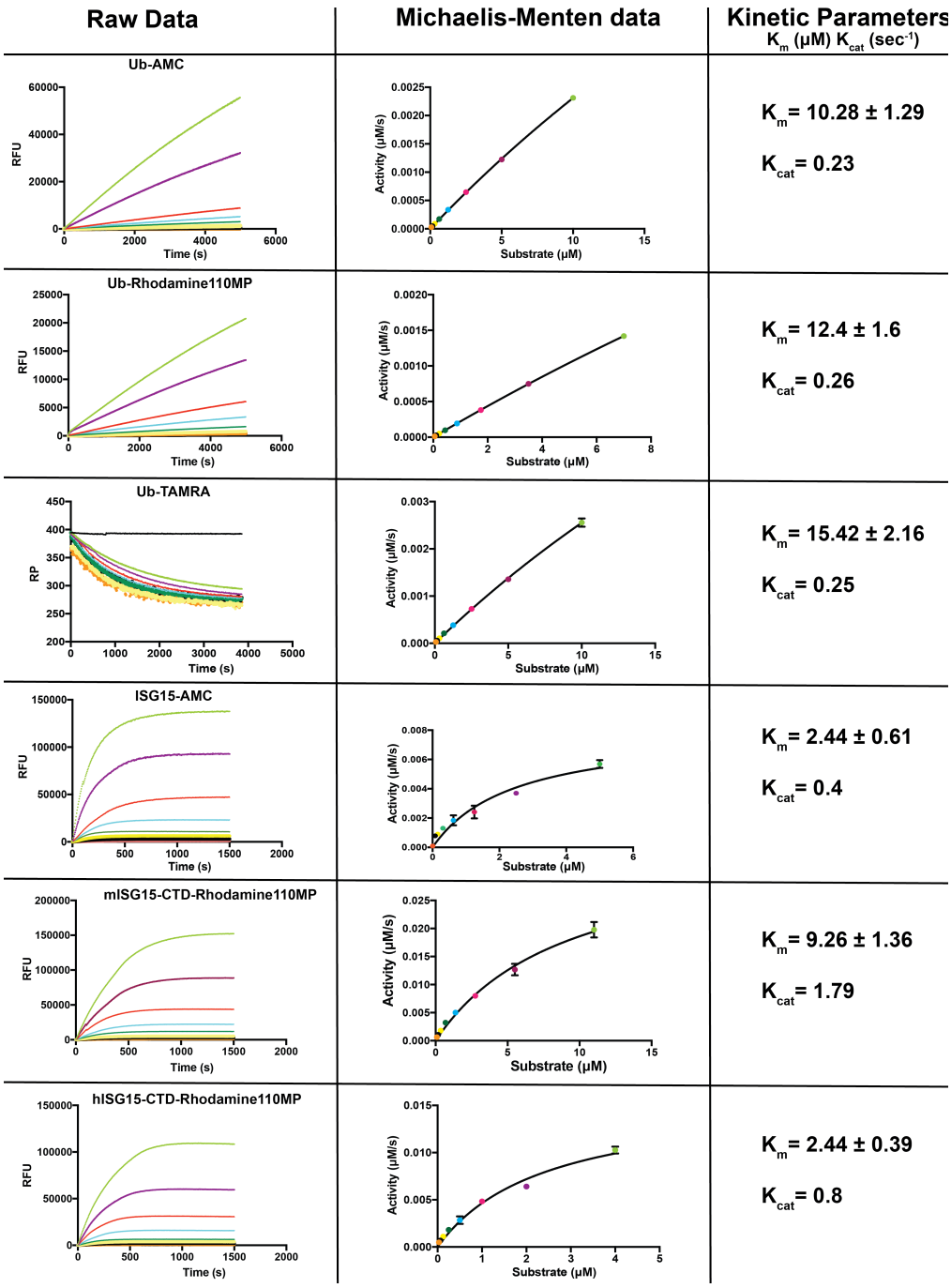


Figure 4. Summary of results showing Michaelis–Menten kinetic parameters for SARS-CoV-2 PLpro using various fluorescently labeled substrates. Shown here is the plot between relative fluorescence unit (RFU) and relative polarimetry (RP) with time in seconds, termed as "raw data." Each color represents substrate concentration. Using the raw data, the Michaelis–Menten graph was plotted to calculate kinetic parameters using GraphPad software.

4 Notes

1. When using sonication to isolate proteins, optimizing the sonication cycles/amplitude is recommended since over-sonication can irreversibly oxidize the active cysteine of PLpro and affect the ABPs-mediated conjugation. Moreover, the use of untagged PLpro from SARS-CoV-1 and SARS-CoV-2 is advised to maintain consistency when comparing activity.
2. It is crucial to reconstitute the probes in the lyophilized powder form according to the manufacturer's protocol, especially the order of solvent added to make the stock solutions. If this reconstitution is not done properly, the probes may precipitate.
3. If the probes precipitate (e.g., due to a high concentration), spin the solution at high speed and collect the supernatant. Next, run a SDS-PAGE gel with a standard protein (such as ubiquitin) to estimate the concentration of the remaining probe solution.
4. The reaction can be performed on ice, but it is recommended to do the assay in a cold room to avoid temperature fluctuations. The assay can be performed at room temperature, but shorter time points are advised (see Note 7).
5. It is recommended to keep the molar ratio between PLpro and probe at 1:3. If there is no limitation of the probe, a higher ratio like 1:5 can be tested.
6. After the addition of the probe to initiate the reaction, it is advised to flick the tube and then start the timer to ensure the probe is evenly mixed in the micro-centrifuge tube.
7. These time points were chosen based on how fast all the PLpro protein was modified by the probe. If one needs to go for shorter time points for sensitive experiments like kinetic analysis, it is recommended to use a quench-flow or stop-flow instrument, which is an automated instrument used to quench enzymatic reactions at a millisecond timescale.
8. The gel can be stained using SYPRO Ruby protein gel stain for a more sensitive reading depending on the need.
9. The kinetic assays can be performed at higher temperatures (e.g., 30–37 °C). However, this will increase the evaporation rate, which might affect the data if the incubation time is long. Maintaining the proteins and performing the experiment at room temperature makes it straightforward to replicate. Hence it is recommended to carry out the assay at room temperature.
10. Important: Please use a black deep well and not a white assay plate for doing the experiment as it has low background fluorescence, less light scattering, and reduced signal crosstalk. It is also recommended to use alternate wells of the plate for the assay as it prevents accidental spillage of the probe and works well with a typical 8 or 12 multichannel pipette (for 384-well plate).
11. Care should be taken that the probes are mixed thoroughly before being dispensed to the next well, and air bubbles are avoided.

12. Another way to plot a standard curve is to use free fluorophore, i.e., not attached to a protein. Specific dilutions of the fluorophore can be prepared in the 1× assay buffer and readings taken on a plate reader. The data can be processed as explained in the Data Analysis section.
13. It is necessary to keep the focal height for all experiments identical, including standard curve calculation, for consistent data analysis and comparison. Therefore, adding the minimum volume of the concentrated enzyme would not change the height considerably.
14. Choose the exact plate if possible because all the focal and gain adjustment calculations depend on this information.
15. Under the drop-down menu, only those filter modules appear which are already placed in the machine. If you do not see the correct optic module, you have to physically put it into the machine.
16. Important: The focal height and gain adjustment values are crucial and should be kept constant as much as possible to get relevant and consistent data. These values depend on the fluorescent probe type, the volume of the sample, the selected optic module, and the plate type. Therefore, keeping a consistent set of values for focal height and gain for a specific fluorescent probe is recommended.
17. Before clicking on "measure one plate," check one last time on the top right of the page whether the right optic module is selected.
18. The "no. of cycles" is a crucial parameter and decides how long the data collection will run for the kinetic assay. One can try to optimize the reaction time, by varying the number of cycles, and click on "check timing" tab on the same page to see the exact duration. If the number of probes is limited and you want to save time on optimization, it is recommended to run the collection for a longer time period, and once the signal reaches saturation, stop the run. The data will be saved until that time. The "cycle time" takes on a default value and depends on how many wells you select on the "layout window."
19. For a specific probe, the values for focal height and gain adjustment should be kept the same for both "Endpoint mode" and "Plate mode." This detail is very important since only then the standard graph generated from "Endpoint mode" will correlate with the kinetic data from "Plate mode."
20. An R-square value of close to 1 is expected if the fluorescence readings are in the linear range.
21. Data normalization can also be done using the division method, but this leads to loss of signal, and the data generated from the standard curve cannot be correlated to it. Hence it is recommended to use the subtraction method.
22. It is recommended to use the linear range of the graph to calculate the slope. All calculations of the kinetic parameters depend on this.
23. If the graph plotted between the rate of reaction and substrate concentration does not follow Michaelis–Menten equation and instead forms a straight line, then the slope of the graph gives K_{cat}/K_m value. It is difficult to obtain the K_m and K_{cat} individually.

UbiQ
targeting the ubiquitin system

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