SARS-CoV-2 3CLpro and PLpro
Luminescent Assays
Application Note

Luminescent Protease Assays:

Luminescent protease assays employ peptide aminoluciferin (peptide-aLuc) substrates in a homogeneous format that is ideally configured in multi-well plates and used for screening and characterizing protease inhibitors. In a 1st reaction a protease cleaves a substrate peptide moiety to release aminoluciferin (aLuc) that accumulates and drives a 2nd reaction with a luciferase that produces light in proportion to protease activity (Fig.1). The system uses a highly stabilized luciferase (UltraGlo™ Luciferase) in a Luciferin Detection Reagent that produces glow-style luminescence with a typical $t_{1/2} \geq 2$ hours.

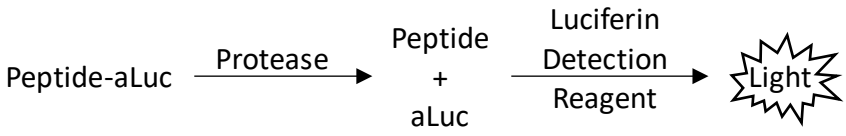
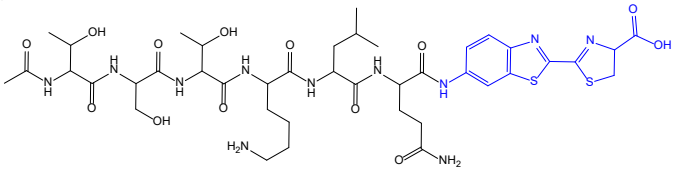


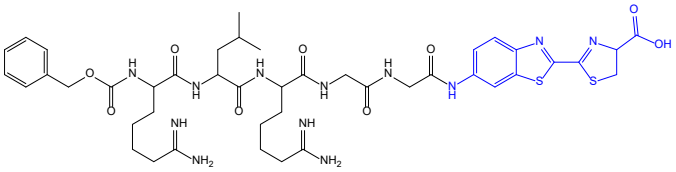
Figure 1. Luminescent protease assay scheme. Protease and test article or vehicle are combined in an opaque white multi-well plate and a reaction is initiated by addition of a peptide-aLuc substrate. The reaction is stopped, and luminescence is initiated by adding Luciferin Detection Reagent. Signal is recorded on a plate-reading luminometer. Inhibitors are identified as test articles that reduce light output.

SARS CoV-2 3CLpro and PLpro luminescent assays:

3CLpro (a.k.a. Main Protease or Mpro) is a chymotrypsin-like protease and PLpro a papain-like protease. Both are encoded in the SARS-CoV-2 genome and play essential roles in the lifecycle of this virus. With the aLuc moiety shown in blue, the luminogenic 3CLPro Substrate is Ac-TSTKLQ-aLuc:



and the luminogenic PLpro Substrate is Z-RLRGG-aLuc:



Figures 2-4 show data from reactions of either recombinant SARS-CoV-2 3CLpro with Ac-TSTKLQ-aLuc or recombinant SARS-CoV-2 PLpro with Z-RLRGG-aLuc.

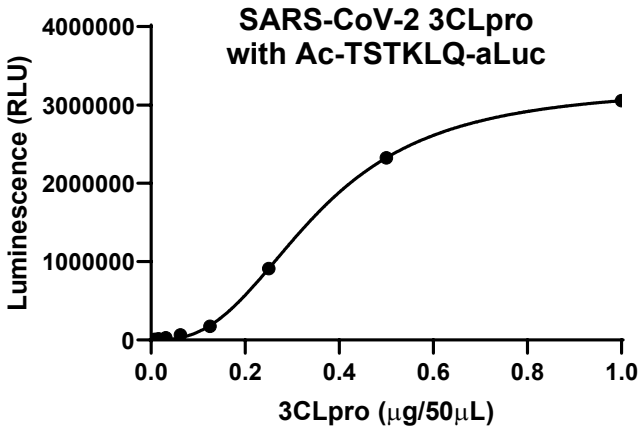


Figure 2. SARS-CoV-2 3CLpro enzyme assay with Luminogenic Substrate. 3CLpro (BPS, Cat# 100823) was combined with 20μM Ac-TSTKLQ-aLuc, 50mM HEPES (pH 7.2), 10mM DTT, and 0.1mM EDTA in a 50μl reaction volume in an opaque white 96-well plate and incubated for 1 hour at 37°C. Reactions were then terminated by adding 50μL of Luciferin Detection Reagent (Promega Cat.# V8920/V8921) and after 20 minutes at room temperature (20° – 25°C) luminescence was recorded on a GloMax® luminometer (Promega Cat.# GM2000). See step-by-step test compound screening protocol at the end of this document.

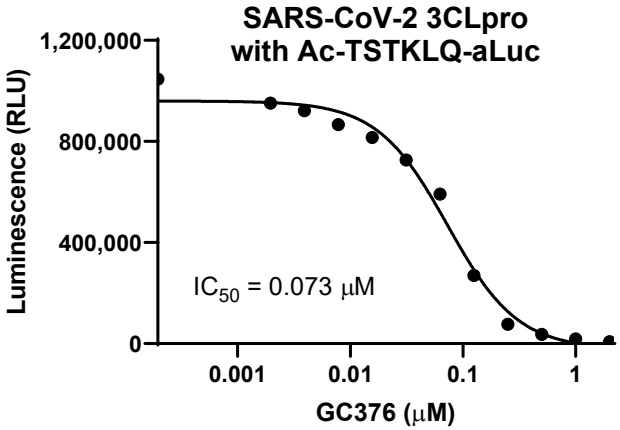


Figure 3. Detection of SARS-CoV-2 3CLpro inhibition with luminescent assay. 0.2μg of untagged* SARS-CoV-2 3CLpro (BPS Bioscience cat# 100823) was combined with 20μM Ac-TSTKLQ-aLuc, the 3CLpro inhibitor GC376 (AmBeed Cat.# A723661, diluted from 4mM stock DMSO solution; see Gurard-Levin et al Antiviral Research 182 (2020) 104924), 50mM HEPES (pH 7.2), 10mM DTT, and 0.1mM EDTA in a 50μl reaction volume in an opaque white 96-well plate, and incubated for 60 minutes at 37°C. Reactions were then terminated by adding 50μL of Luciferin Detection Reagent (Promega Cat.# V8920/V8921) and after 20 minutes at room temperature (20° – 25°C) luminescence was recorded on a GloMax® luminometer (Promega Cat.# GM2000). See step-by-step test compound screening protocol at the end of this document.

*Note: A 6His-tagged preparation of purified recombinant SARS-CoV-2 3CLpro that we tested displayed <1% of the activity of this untagged version and was insensitive to various reported 3CLpro inhibitors. This was consistent with a previous report that affinity tags depressed the activity of the highly homologous SARS-CoV 3CLpro (Grum-Tokers et al, Virus Res 133 (2008) 63–73).

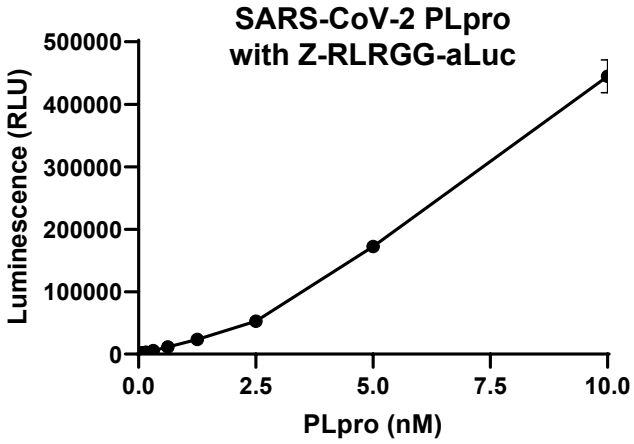


Figure 4. SARS-CoV-2 PLpro enzyme assay with Luminogenic Substrate. GST-PLpro (R&D Systems, Cat# E-611-050) was combined with 20μM Z-RLRGG-aLuc, 50mM HEPES (pH 7.2), 10mM DTT, and 0.1mM EDTA in a 50μl reaction volume in an opaque white 96-well plate and incubated for 30 minutes at 20° – 25°C. Reactions were then terminated by adding 50μL of Luciferin Detection Reagent (Promega Cat.# V8920V8921) and after 20 minutes at room temperature (20° – 25°C) luminescence was recorded on a GloMax® luminometer (Promega Cat.# GM2000). See step-by-step test compound screening protocol at the end of this document.

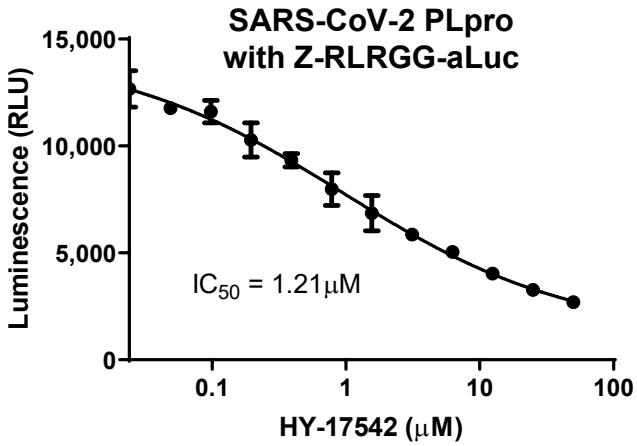


Figure 5. Detection of SARS-CoV-2 PLpro inhibition with luminescent assay. 0.5nM SARS-CoV-2 GST-PLpro (R&D Systems, Cat# E-611-050) was combined with 20μM Z-RLRGG-aLuc, the PLpro inhibitor HY-17542 (MedChemExpress, diluted from 100mM stock DMSO solution; Loffredo et al https://doi.org/10.1101/2020.07.15.203059), 50mM HEPES (pH 7.2), 10mM DTT, and 0.1mM EDTA in a 50μl reaction volume in an opaque white 96-well plate, and incubated for 30 minutes at 20° – 25°C. Reactions were then terminated by adding 50μL of Luciferin Detection Reagent (Promega Cat.# V8920/V8921) and after 20 minutes at room temperature luminescence was recorded on a GloMax® luminometer (Promega Cat.# GM2000). See step-by-step test compound screening protocol at the end of this document.

The luminogenic SARS-CoV-2 protease substrates are currently available as early access materials from Promega along with the Promega catalogue items described in this Application Note as follows:

Product	Size	Cat. #
Luciferin Detection Reagent	10ml	V8920
Luciferin Detection Reagent	50ml	V8921
Luminogenic PLpro Substrate		please enquire*
Luminogenic 3CLpro Substrate		please enquire*
GloMax® Navigator Microplate Luminometer		GM2000

*Michael Curtin, Ph.D., Product Manager
Email: michael.curtin@promega.com
Phone: 608 298 4650

Step-by-step compound screening protocols

SARS-CoV-2 PLpro Assay

Materials

- The PLpro substrate is supplied at 4mM in 0.5M HEPES, pH 7.2 as a custom material from Promega Corp. Please inquire:
  Michael Curtin, Ph.D., Product Manager
  Email: michael.curtin@promega.com
  Phone: 608 298 4650
- Assay buffer: 50mM HEPES pH 7.2, 10mM DTT, and 0.1mM EDTA (prepared by user)
- Luciferin Detection Reagent (Promega Cat.# V8920 or V8921)
- Recombinant PLpro sources:
  - R&D Systems Cat.# E-611-050
  - AcroBiosystems Cat.# PAE-C5148
- Opaque white 96 well plates (e.g., Corning Cat.# 3912)
- Plate reading luminometer (e.g., Promega GloMax, Cat.# GM2000)

Preparing reagents:

- Prepare 2X PLpro substrate solution at RT°: Dilute PLpro substrate to 40µM in assay buffer.
- Prepare 4X PLpro enzyme solution on ice: Dilute PLpro recombinant enzyme to 2nM in assay buffer (see Figure 3 to consider enzyme concentration adjustments).
- Prepare 4X test compound solutions in assay buffer at RT°.
- Prepare Luciferin Detection Reagent at RT°: this reagent is supplied in 2 components, a lyophilized preparation, and a reconstitution buffer.
  - Add the entire contents of the reconstitution buffer to the Luciferin Detection Reagent lyophilized cake. Mix thoroughly but gentle to avoid forming bubbles (store unused portion at -20°C).

Performing the assay:

1. Dispense 25µL 2X PLpro substrate solution into an opaque white 96-well plate.
2. Add 12.5µL 4X test compound solutions to substrate solution in the 96-well plate.

3. Initiate reactions by adding 12.5µL 4X PLpro solution to substrate solution in the 96 well plate.
4. Mix plate and incubate for 30 minutes at room temperature (20°-25°C)
5. Add 50µL Luciferin Detection Reagent to each well to stop reactions and initiate luminescent signals. Allow 10 minutes for signal stabilization.
6. Read luminescence on a plate reading luminometer (e.g. Promega GloMax, Cat.# GM2000)

SARS-CoV-2 3CLpro Assay

Materials

- The 3CLpro substrate is supplied at 4mM in 0.5M HEPES, pH 7.2 as a custom material from Promega Corp. Please inquire:
  Michael Curtin, Ph.D., Product Manager
  Email: michael.curtin@promega.com
  Phone: 608 298 4650
- Assay buffer: 50mM HEPES pH 7.2, 10mM DTT, and 0.1mM EDTA (prepared by user)
- Luciferin Detection Reagent: Promega Cat.# V8920 or V8921
- Recombinant 3CLpro: BPS Bioscience Cat.# 100823
- Opaque white 96 well plates (e.g., Corning, Cat.# 3912)
- Plate reading luminometer (e.g., Promega GloMax, Cat.# GM2000)

Preparing reagents:

- Prepare a 2X 3CLpro substrate solution (40µM) at RT°: Dilute 4mM 3CLpro substrate to 40µM in assay buffer.
- Prepare 4X 3CLpro enzyme solution on ice: Dilute 3CLpro untagged recombinant enzyme to 16µg/mL in assay buffer (see Figure 2 to consider enzyme concentration adjustments).
- Prepare 4X test compound solutions in assay buffer at RT°.
- Prepare Luciferin Detection Reagent at RT°: this reagent is supplied in 2 components, a lyophilized preparation, and a reconstitution buffer.
  - Add the entire contents of the reconstitution buffer to the Luciferin Detection Reagent lyophilized cake. Mix thoroughly but gentle to avoid forming bubbles (store unused portion at -20°C).

Performing the assay:

1. Dispense 25µL 2X 3CLpro substrate solution into an opaque white 96-well plate.
2. Add 12.5µL 4X test compound solutions to substrate solution in the 96-well plate.
3. Initiate reactions by adding 12.5µL 4X 3CLpro enzyme solution to substrate solution in the 96 well plate.
4. Mix plate and incubate for 1 hour at 37°C.
5. Add 50µL Luciferin Detection Reagent to each well to stop reactions and initiate luminescent signals. Allow 20 minutes for signal stabilization.
6. Read luminescence on a plate reading luminometer (e.g., Promega GloMax, Cat.# GM2000).