SensoLyte® 490 HIV-1 Protease Assay Kit
Fluorimetric

Revision# 1.2	*Last Updated: July 2021*
Catalog #	**AS-71127**
Kit Size	500 Assays (96-well plate)

- ***Convenient Format:*** Complete kit including all the assay components.
- ***Optimized Performance:*** Optimal conditions for the detection of HIV-1 protease activity.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references was provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	HIV-1 protease substrate EDANS/DABCYL FRET peptide Ex/Em=340nm/490nm upon cleavage	600 µL
Component B	EDANS, fluorescence reference standard	100 µM DMSO solution, 20 µL
Component C	Pepstatin A	27.4 µg powder
Component D	2X Assay buffer	50 mL
Component E	Stop solution	30mL
Component F	DMSO	100 µL
Component G	DTT	1 M, 200 µL

Other Materials Required (but not provided)

- HIV-1 protease: HIV-1 protease can be produced either from *E. coli* or by chemical synthesis. AnaSpec provides active recombinant HIV-1 protease (Cat#72028-5)
- 96-well microplate: Black, flat bottom plate with non-binding surface
- Fluorescence microplate reader: Capable of excitation at 340±30 nm and emission at 490±30 nm

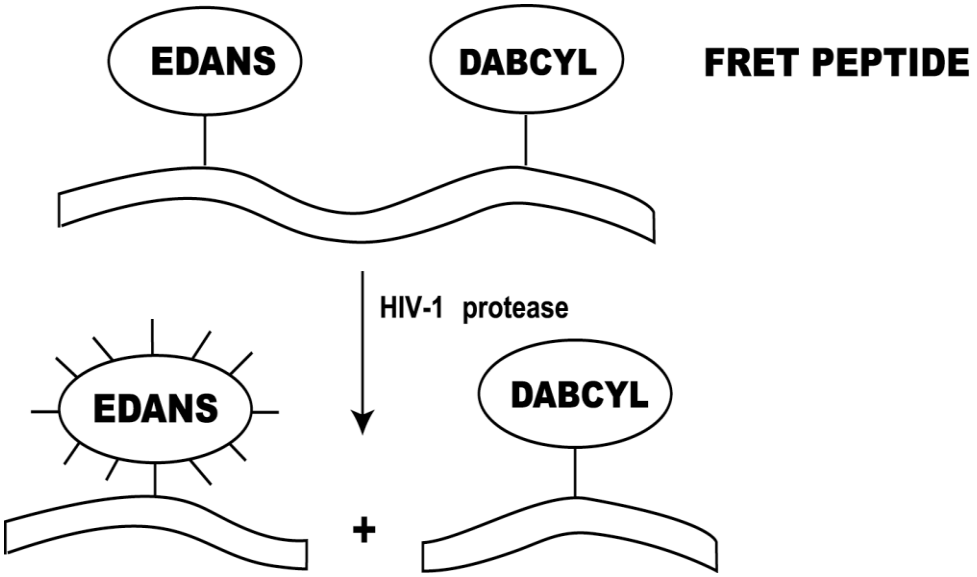
Storage and Handling

- Store all kit components at -20°C
- Protect Components A and B from light

Introduction

The 10~12 kD aspartic protease of human immunodeficiency virus-1 (HIV-1) is required for the post-translational cleavage of the precursor polyproteins, Pr^{gag} and $Pr^{gag\text{-}pol}$.[1] These cleavages are essential for the maturation of HIV infectious particles. Thus, the protease becomes one of the key targets for developing anti-AIDS drugs.

The SensoLyte® 490 HIV-1 Protease Assay Kit provides a convenient assay for high throughput screening of HIV-1 protease inhibitors and continuous quantification of HIV-1 protease activity using a fluorescence resonance energy transfer (FRET) peptide. The sequence of this FRET peptide is derived from the native p17/p24 cleavage site on Pr^{gag} for HIV-1 protease. In the FRET peptide, the fluorescence of EDANS is quenched by DABCYL until this peptide is cleaved into two separate fragments by HIV-1 protease at the Tyr-Pro bond (see **Scheme 1**). Upon cleavage, the fluorescence of EDANS is recovered, and can be monitored at excitation/emission = 340 nm/490 nm.



Scheme 1. Proteolytic cleavage of EDANS/DABCYL FRET peptide by HIV-1 protease.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Warm up all the kit components until thawed at room temperature before starting the experiments.
Please choose Protocol A or B based on your needs

Protocol A. Screening HIV protease inhibitors using purified HIV-1 protease

1. Prepare working solutions.

1.1 1X Assay buffer : Prepare 1X assay buffer according to Table 1. Prepare this DTT-containing 1X assay buffer fresh for the experiment.

Table 1. 1X Assay buffer for one 96-well plate (100 assays).

Components	Volume
2X assay buffer (Component D)	5 mL
1 M DTT (1000X, Component G)	10 µL
Deionized water	5 mL
Total volume	10 mL

1.2 HIV-1 protease substrate solution: Prepare HIV-1 protease substrate solution according to Table 2. Mix the reagents well. Prepare fresh substrate solution for the experiment.

Table 2. HIV-1 protease substrate solution for one 96-well plate (100 assays).

Components	Volume
HIV-1 protease substrate (50X,Component A)	100 µL
1X Assay buffer	4.9 mL
Total volume	5 mL

1.3 HIV-1 protease diluent: Dilute HIV protease to an appropriate concentration in assay buffer. The recommended volume for HIV-1 protease diluent is 40 µL/well. You may adjust the volume according to your preference.

Note: Prepare enzyme diluent immediately before use. Avoid vortexing enzyme. Prolonged storage of diluted enzyme or vigorously vortexing will denature the enzyme. Keep enzyme on ice.

1.4 Test compound: Dilute test compounds with deionized water or an appropriate vehicle. The recommended volume for the diluted test compound is 10 µL/assay. You may adjust the volume according to your preference.

1.5 Pepstatin A[4] (control inhibitor): Add 20 µL of DMSO (Component F) into one vial of Pepstatin A (Component C) to get a concentration of 2 mM. Dissolve it completely by vortexing. Dilute 2 mM Pepstatin A to 2 µM in assay buffer. Prepare 10 µL per well of 2 µM pepstatin A.

Note: 2 mM Pepstatin A should be stored at -20°C for future use. 2 µM Pepstatin A is not stable, and should be prepared fresh for each experiment.

2. Set up enzymatic reaction.

2.1 Add test compounds and HIV-1 protease diluent into microplate. The suggested total volume of HIV-1 protease diluent plus test compound is 50 µL, for example 40 µL of protease diluent and 10 µL of test compound. You may adjust the volume according to your preference.

2.2 Set up the following controls at the same time:

- Positive control contains HIV-1 protease diluent without test compound.
- Inhibitor control contains HIV-1 protease diluent and known inhibitor, pepstatin A (10 µL/well).
- Vehicle control contains HIV-1 protease diluent and vehicle used to deliver test compound (e.g. DMSO).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 µL.

3. Pre-incubation.

3.1 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. Also incubate the HIV-1 protease substrate solution at the same temperature.

4. Initiate the enzymatic reaction.

4.1 Add 50 µL per well of HIV-1 protease substrate solution. Mix the reagents completely by shaking the plate gently for 30-60 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=340±30 nm/490±30 nm continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 min. Keep the plate from direct light. Optional: Add 50 µL/well of stop solution (Component E). Mix the reagents. Then measure fluorescence intensity at Ex/Em=340±30 nm/490±30 nm.

Note: If the stop solution looks cloudy, warm in a 37°C water bath to dissolve the precipitate.

4.3 Data analysis: Refer to Appendix I.

Protocol B. Measuring HIV-1 protease activity in biological samples.

1. Prepare working solutions.

1.1 HIV-1 protease substrate solution: Prepare HIV-1 protease substrate solution according to Table 3. Mix the reagents well. Prepare fresh substrate solution for the experiment.

Table 3. HIV-1 protease substrate solution for one 96-well plate (100 assays).

Components	Volume
HIV-1 protease substrate (50X, Component A)	100 µL
1 M DTT (1000X, Component G)	5 µL
2X Assay buffer (Component D)	4.9 mL
Total volume	5 mL

2. Set up enzymatic reaction.

2.1 Add 50 µL/well of HIV-1 protease containing biological sample.

2.2 Simultaneously set up the following controls:

- Positive control contains HIV-1 protease positive sample.
- Negative control contains biological sample without HIV-1 protease.
- Substrate control contains deionized water.

2.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 µL.

3. Initiate the enzymatic reaction.

3.1 Add 50 µL/well of HIV-1 protease substrate solution. Mix the reagents completely by shaking the plate gently for 30-60 sec.

3.2 Measure fluorescence signal:

- For kinetics reading: Immediately start measuring fluorescence intensity at Ex/Em=340±30 nm/490±30 nm continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 min. Keep the plate from direct light. Optional: Add 50 µL/well of stop solution (Component E). Then measure fluorescence intensity at Ex/Em=340±30 nm/490±30 nm.

3.3 Data analysis: Refer to Appendix I.

Appendix I: Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells. This reading is the relative fluorescence unit (RFU).
- For kinetics reading:
  - Plot data as RFU versus time for each sample. To convert RFU to the concentration of the product of enzymatic reaction, please refer to Appendix II for setting up the fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
  - Obtain the initial reaction velocity (Vo) in RFU/min. Determine the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, K_m, K_i, etc.
- For endpoint reading:

- Plot data as RFU versus the concentration of test compounds.
- A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II: Instrument Calibration

- EDANS fluorescence reference standard: Dilute 100 µM EDANS (Component B) to 1 µM in 1X assay buffer (refer to Protocol A Step 1.1 for preparation). Do 2-fold serial dilutions to get concentrations of 500, 250, 125, 62.5, 31.25, and 15.63 nM, include an assay buffer blank. Add 50 µL/well of these serially diluted EDANS reference standards.
- Add 50 µL/well of HIV-1 protease substrate solution (refer to protocol A step 1.2 for preparation).

  Note: The HIV-1 protease substrate solution should be added to the EDANS reference standard to correct for the fluorescence inner filter effect.
- Optional: If the stop solution (Component E) was added into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to reference standard wells to obtain a better comparison.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=340/490 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot EDANS fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as **Figure 1**.

  Note: The final concentrations of EDANS reference standards are 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 nM. This reference standard curve is used to calibrate the variation of different instruments and the different batches of experiments. It is also an indicator of the amount of final product of the HIV-1 protease enzymatic reaction.

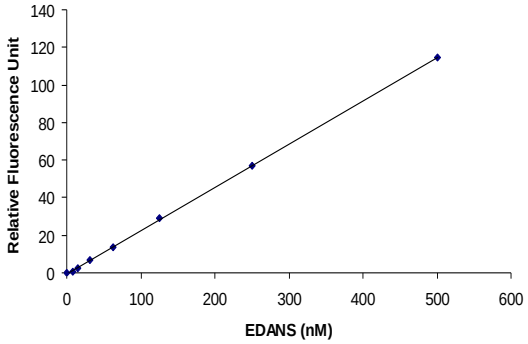


Figure 1. EDANS reference standard calibration curve. EDANS was diluted in assay buffer containing HIV-1 protease substrate. Fluorescence signal was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments) with a filter set of Ex/Em=340±30 nm/490±40

References

1. Seelmeier, S. et al. *Proc.Natl.Acad.Sci.U.S.A* **85**, 6612 (1988).
2. Gehringer, H. et al. *J.Virol.Methods* **109**, 143 (2003).
3. Schneider, J., et al. *Cell* **54**, 363 (1988).