EnzoLyte™ 490 HIV-1 Protease Assay Kit
Fluorimetric

Catalog #	71127
Unit Size	1 Kit
Kit Size	500 Assays (96-well) or 1250 Assays (384-well)

This kit is optimized to detect Human Immunodeficiency Virus-1 (HIV-1) protease activity using an EDANS/DABCYL FRET peptide as a substrate. The fluorescence signal can be monitored at Ex/Em=340 nm/490 nm upon proteolytic cleavage. Ample materials are provided to perform 500 assays in a 96-well format or 1250 assays in a 384-well format. The kit has the following features:

- ***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 are provided.

USA and Canada Ordering Information

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International Ordering Information

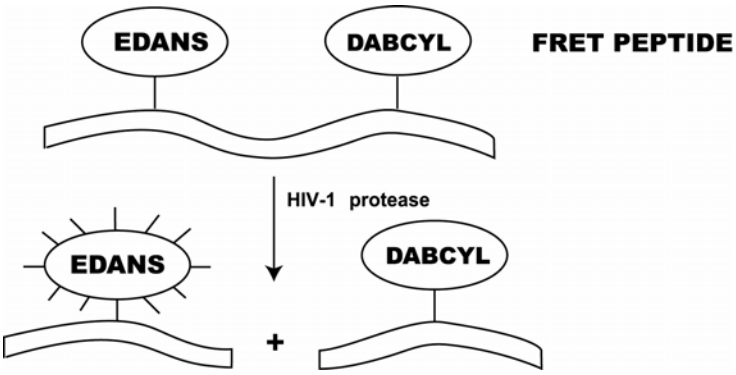
A list of international distributors is available at www.anaspec.com.

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]. Since these cleavages are essential for the maturation of HIV infectious particles, this protease has become one of the key targets for developing anti-AIDS drugs.

The EnzoLyte™ 490 HIV-1 Protease Assay Kit provides a convenient assay for high throughput screening of HIV-1 protease inhibitors and for 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 this FRET peptide, the fluorescence of EDANS is quenched by DABCYL. Upon cleavage into two separate fragments by the HIV-1 protease at the Tyr-Pro bond (see **Scheme 1**), the fluorescence of EDANS is recovered, and can be monitored at excitation/emission = 340 nm/490 nm.

The assays are performed in a convenient 96 or 384-well microplate format.



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

KIT COMPONENTS, STORAGE AND HANDLING

Note: Store all kit components at -20℃, and keep components A and B away from light.

Component A: HIV-1 protease substrate (600 µL)
EDANS/DABCYL FRET peptide, Ex/Em=340nm/490nm upon cleavage

Component B: EDANS, fluorescence reference standard (100 µM DMSO solution, 20 µL)
Ex/Em=340nm/490nm

Component C: Pepstatin A, a characterized HIV-1 protease inhibitor (27.4 µg powder)

Component D: 2X Assay buffer (50 mL)

Component E: Stop solution (30 mL)

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* [2] or by chemical synthesis[3].
96-well microplate: Black microplate provides better signal to noise ratio.
Fluorescence microplate reader: Capable of excitation at 340±30 nm and emission at 490±30 nm.

STANDARD OPERATION PROTOCOL

Note 1: For fluorescence instrument calibration, please refer to Appendix II (recommended for first-time users).

Note 2: Warm all kit components until thawed to 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

- Assay buffer: Dilute 2X assay buffer (component D) to 1X with deionized water. Add 1 M DTT (component G) 1:1000 into 1X assay buffer. Use this DTT-containing 1X assay buffer in ***all*** the following steps.
  Note: Prepare fresh DTT-containing 1X assay buffer for each experiment.
- HIV-1 protease substrate solution: Dilute HIV-1 protease substrate (component A) 1:50 in assay buffer. Mix the reagents well.
  Note: Prepare fresh substrate solution for each experiment.
- HIV-1 protease diluent: Dilute HIV protease to an appropriate concentration in assay buffer.
  Note: Prepare enzyme diluent immediately before use. Do not vortex enzyme. Prolonged storage of diluted enzyme or vigorously vortexing will denature the enzyme. Keep the enzyme on ice.
- Test compound: Dilute test compounds with deionized water or an appropriate vehicle.
- 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 completely by vortexing. Dilute 2 mM Pepstatin A to 2 µM in assay buffer.

  Note: 2 mM Pepstatin A should be stored at -20℃ for further use. It is not stable, and should be prepared fresh for each experiment.

2. Set up enzymatic reaction

- Add test compounds and HIV-1 protease diluent into a microplate. The suggested total volume of HIV-1 protease diluent and test compound for a 96-well plate is 50 µL. The suggested total volume of HIV-1 protease and test compound for a 384-well plate is 20 µL.
- 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 for 96-well plate or 4 µL for 384-well plate).
  - 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.

Note: Bring the total volume of all the controls to 50 µL/well for a 96-well plate or 20 µL/well for a 384-well plate with assay buffer.

3. Pre-incubation

- Incubate the plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. In the meantime, also incubate the HIV-1 protease substrate solution at the same temperature.

4. Initiate the enzymatic reaction

- Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HIV-1 protease substrate. Mix the reagents completely by shaking the plate gently for 30-60 seconds.
- 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 minutes for 30 to 60 minutes.

  For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate away from direct light. (Optional) add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of stop solution (component E). Mix the reagents, then measure fluorescence intensity at Ex/Em=340±30 nm/490±30 nm.
- Data analysis: Refer to Appendix I.

Protocol B Measuring HIV-1 protease activity in biological samples

1. **Prepare working solutions**
   - 2X Assay buffer: Add 1 M DTT (component G) 1:500 into 2X assay buffer (component D). Use this DTT-containing 2X assay buffer in ***all*** the following steps.
     Note: Prepare fresh DTT -containing assay buffer for each experiment.
   - HIV-1 protease substrate solution: Dilute HIV-1 protease substrate (component A) 1:50 in 2X assay buffer. Mix the reagents well.
     Note: Prepare fresh substrate solution for each experiment.
2. **Set up enzymatic reaction**
   - Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HIV-1 protease containing biological sample.
   - Set up the following controls at the same time:
     - Positive control contains HIV-1 protease standard.
     - Negative control contains biological sample without HIV-1 protease.
     - Substrate control contains deionized water.

   Note: Bring the total volume of all the controls to 50 µL/well for a 96-well plate or 20 µL/well for a 384-well plate.
3. **Initiate the enzymatic reaction**
   - Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HIV-1 protease substrate. Mix the reagents completely by shaking the plate gently for 30-60 seconds.
   - 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 minutes for 30 to 60 minutes.

     For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate away from direct light. (Optional) add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of stop solution (component E). Mix the reagents, then measure fluorescence intensity at Ex/Em=340±30 nm/490±30 nm.
   - Data analysis: Refer to Appendix I.

Appendix I: Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. Subtract this background reading from the readings of the other wells to get the relative fluorescence unit (RFU).
- For kinetics reading:
  - Plot data as RFU versus time for each sample. To convert RFU to concentration of the product of enzymatic reaction, please refer to Appendix II on how to set 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 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 deionized water. Perform 1:2 serial dilutions to get concentrations of 500, 250, 125, 62.5, 31.25, 15.63 and 0 nM. Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of the serially diluted EDANS from 1 μM to 0 nM.
- Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of HIV-1 protease substrate solution (refer to protocol B step 1 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 the reference standard wells to obtain a better comparison.
- Plot EDANS fluorescence reference standard as RFU (relative fluorescence unit) versus concentration as in **Figure 1**.

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

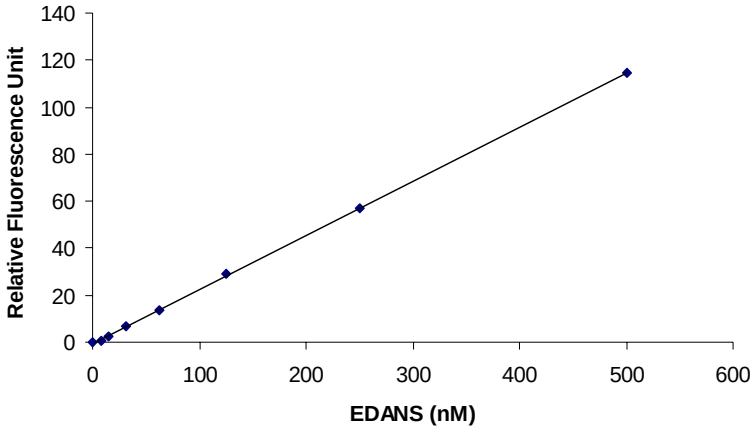


Figure 1. The EDANS reference standard calibration curve.

EDANS was diluted in assay buffer containing HIV-1 protease substrate. 100 μL of EDANS at each concentration was added into a black 96-well microplate. The fluorescence signal was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments) with a filter set of Ex/Em=360±40 nm/460±40 nm. (Samples were done in duplicates).

REFERENCES

1. S. Seelmeier, H. Schmidt, V. Turk, H. K. von der, *Proc.Natl.Acad.Sci.U.S.A* 85, 6612-6616 (1988).
2. H. Gehringer et al., *J.Virol.Methods* 109, 143-152 (2003).
3. J. Schneider and S. B. Kent, *Cell* 54, 363-368 (1988).
4. S. Seelmeier, H. Schmidt, V. Turk, H. K. von der, *Proc.Natl.Acad.Sci.U.S.A* 85, 6612-6616 (1988).