HIV Protease Fluorimetric Assay Kit

Cat. No. KITG-027 **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The 10-12 kD aspartic protease of human immunodeficiency virus-1 (HIV-1) is required for the posttranslational cleavage of the precursor polyproteins, Prgag and Prgag-pol. 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.
Species	Human Immunodeficiency Virus-1 (HIV-1)
Description	This 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 Prgag for HIV-1 protease. In the FRET peptide, the fluorescence of the donor is quenched by the acceptor until this peptide is cleaved into two separate fragments by HIV-1 protease. Upon cleavage, the fluorescence of the donor is recovered, and can be monitored at excitation/emission = 490 nm/520 nm. With excellent fluorescence quantum yield and longer excitation and emission wavelength, the fluorescence signal of the donor is less interfered by the autofluorescence of cell components and test compounds. The assay can detect as low as 250 ng/mL active HIV-1 protease.
Form	Fluorimetric Activity Assay Kit
Applications	High throughput screening of HIV-1 protease inhibitors and continuous quantification of HIV-1 protease activity. For research use only. Not for use in diagnostic

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	procedures.
Notes	Kit Components: Component A: HIV-1 protease FRET substrate, 120 µL, store at -20°C, protect from light and moisture. Component B: Fluorescence reference standard, 27.4 µg powder, store at -20°C, protect from light and moisture. Component C: Pepstatin A (characterized HIV-1 protease inhibitor), store at -20°C. Component D: 2X Assay buffer, 20 mL, store at -20°C. Component E: Stop solution, 10 mL, store at -20°C. Component F: DMSO, 50 µL, store at -20°C. Component G: DTT, 1 M, 150 µL, store at -20°C. Other Materials Required (not provided): - HIV-1 protease: HIV-1 protease can be produced either from E. coli or by chemical synthesis. - 96-well microplate: Black, flat-bottom plate with non-binding surface. - Fluorescence microplate reader: Capable of excitation at 490 nm and emission at 520 nm. Storage and Handling: Store all kit components at -20°C. Protect Components A and B from light and moisture. Protocol A: Protocol A: Screen HIV protease inhibitors using purified HIV-1 protease Note 1: For instrument calibration, refer to Appendix II. Note 2: Warm up all the kit components until thawed at room temperature before starting the assay. Note 3: Please choose Protocol A or B based on your application. 1. Prepare working solutions: 1.1 1X Assay buffer: Prepare 1X assay buffer according to the following table. Prepare this DTT-containing 1X assay buffer fresh for each assay. Components for one 96-well plate (100 assays): - 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 the following table. Mix the reagents well. Prepare fresh substrate solution for each assay. Components for one 96-well plate (100 assays): - 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 appropriate concentration in 1X assay buffer. The recommended volume for HIV-1 protease diluent is 40 µL/assay (96-well plate). You may adjust the volume according to your needs. Note: Prepare enzyme diluent right before use. Do not vortex enzyme.

Prolonged storage of diluted enzyme or vigorously vortexing will denature the enzyme. Preserve the enzyme on ice. 1.4 Test compound: Dilute test compounds with deionized water or appropriate vehicle. The recommended volume for diluted test compound is 10 µL/assay (96-well plate). You may adjust the volume according to your needs. 1.5 Pepstatin A (control inhibitor): Add 20 µL of DMSO (Component F) into one vial of Pepstatin A (Component C) to get a concentration of 2 mM. Vortex to dissolve it completely. Dilute 2 mM Pepstatin A to 2 µM in deionized water. Prepare 10 µL/well of 2 µM pepstatin A. Note: The 2 mM Pepstatin A should be stored at -20°C for further use. The 2 µM Pepstatin A is not stable, and should be prepared freshly for each assay. 2. Set up enzymatic reactions: 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 needs. 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. - 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 positive results. - Substrate control: contains assay buffer. 2.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 µL. 2.4 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. In the meantime, also incubate the HIV-1 protease substrate solution at the same temperature. 3. Initiate the enzymatic reaction: 3.1 Add 50 µL per well of HIV-1 protease substrate solution. Mix the reagents completely by shaking the plate gently for 30-60 s. 3.2 Measure fluorescence signal: - For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em = 490 nm/520 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 from direct light. Optional: Add 50 µL/well of stop solution (Component E). Mix

the reagents. Measure fluorescence intensity at Ex/Em = 490 nm/520 nm. Note: If stop solution looks cloudy, warm it up in 37°C water bath to dissolve the precipitate. 3.3 Data analysis: Refer to Appendix I. Protocol B: Protocol B: Measure 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 the following table. Mix the reagents well. Prepare fresh substrate solution for each assay. Components for one 96-well plate (100 assays): - 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 reactions: 2.1 Add 50 µL/well of HIV-1 protease containing biological sample. 2.2 Set up the following controls at the same time: - Positive control: contains HIV-1 protease positive control. - 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 s. 3.2 Measure fluorescence signal: - For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em = 490 nm/520 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 from direct light. Optional: Add 50 µL/well of stop solution (Component E). Measure fluorescence intensity at Ex/Em = 490 nm/520 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 kinetic reading: - Plot data as RFU versus time for each sample. If you want to convert the RFU to the concentration of the product of enzymatic reaction, refer to Appendix II for setting up 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 %, EC50, IC50, Km, Ki, etc. For endpoint reading: - Plot data as RFU versus the concentration of test compound. - A variety of data analyses can be done, e.g., determining inhibition %, EC50, IC50, etc. Appendix II: Instrument Calibration - Fluorescence reference standard: Dilute 100 µM fluorescence reference standard (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 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 fluorescence reference standard to correct the fluorescence inner filter effect. - Optional: If the stop solution (Component E) was added into the enzymatic reaction before taking the endpoint reading, the same volume of stop solution should be added to reference standard wells for better comparison. - Measure the fluorescence intensity of the reference standard wells at Ex/Em = 490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme assay. - Plot fluorescent reference standard as RFU (relative fluorescent unit) versus concentration. Note: The final concentrations of fluorescence reference standards are 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 nM. This reference standard is used to calibrate the variation of different instruments and different batch of experiments. It is also an indicator of the amount of final product of the HIV-1 protease enzymatic reaction.
Storage	Store all kit components at -20°C. Protect Components A and B from light and moisture.
Shipping	Shipped on dry ice or blue ice.
EC	2X Assay buffer (Component D), prepare 1X with DTT and deionized water

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Storage Buffer	2X Assay buffer (Component D), prepare 1X with DTT and deionized water
Labeling Efficiency	For research use only. Not for use in diagnostic procedures.
Usage	Pepstatin A (provided as control inhibitor)
Inhibitors	Pepstatin A (provided as control inhibitor)

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