SensoLyte® 520 FAP (Seprase) Assay Kit *Fluorimetric*

Revision Number: 1.0 Last updated: 24OCT2023

Catalog #	AS-72273
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect FAP enzyme activity.
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well plate format.
- ***High Speed:*** The entire process can be completed in one hour.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	HiLyte Fluor™488/QXL®-520 FAP substrate, Ex/Em=490/520 nm upon cleavage	0.4 mM, 50 µL
Component B	HiLyte Fluor™488, fluorescence reference standard, Ex/Em=490/520 nm	0.4 mM, 10 µL
Component C	Recombinant Human FAP (Seprase)	0.1 mg/mL, 10 µL
Component D	2X Assay Buffer	25 mL
Component E	Inhibitor	10 mM, 10 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat-bottom, 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components at -20°C, except for Component C.
- Store Component C at -80°C. Aliquot as needed to avoid freeze-thaw cycles.
- Protect Components A and B from light and moisture.
- Component D can be stored at room temperature for convenience.

Introduction

FAP (Fibroblast activation protein alpha), also known as Seprase, belongs to the family of serine proteases, and is most similar to DPPIV.[1-5] FAP expression is typically associated with such physiological and pathological processes as embryonic development, wound healing, cancer, arthritis, and fibrosis.[1-3] FAP has both dipeptidyl peptidase enzymatic activity and endopeptidase activity. DPPIV also has dipeptidylpetidase activity, but endopeptidase activity is specific to FAP and hence can be used to develop FAP specific detection methods.

The 520 FAP Assay Kit is an assay for screening of enzyme inhibitors or for continuous assay of FAP activity. This kit utilizes a HiLyte Fluor™488/QXL® FRET substrate, that is cleaved by FAP, but not by DPPIV. Upon cleavage by FAP enzyme, the FRET substrate is separated into two fragments resulting in release of HiLyte Fluor™488 fluorescence which can be monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of HiLyte Fluor™488 is less prone to interference by the autofluorescence of components in biological samples and test compounds, compared to shorter wavelength FRET substrates.

Protocol

Note 1: Please use protocol A or B based on your needs.
Note 2: Keep enzyme (Component C) on ice before use. Warm up the rest of kit components at room temperature until thawed before starting the experiments. Spin down all the vials before opening them to ensure retrieval of adequate volume of liquid in the vials.

Protocol A. Screening compounds using purified enzyme.

1. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments. Component C should be kept on ice after thawing.

1.1 1X assay buffer: Add 10 mL of 2X assay buffer (Component D) to 10 mL of deionized water.

1.2 FAP substrate solution: Dilute FAP substrate (Component A) 100-fold in 1X assay buffer (step 1.1) according to Table 1. For each experiment, prepare fresh substrate solution

Table 1. FAP substrate solution for one 96-well plate (100 assays)

Components	Volume
FAP substrate (Component A)	50 µL
1X assay buffer (step 1.1)	4.95 mL
Total volume	5.0 mL

1.3 FAP enzyme solution: Dilute FAP enzyme (Component C) 400-fold in 1X assay buffer (step 1.1). This amount of enzyme is enough for a full 96-well plate. If not using the entire plate, adjust the amount of enzyme to be diluted accordingly. Note: Prepare enzyme solution immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 FAP inhibitor, (Talabostat mesylate): Dilute the 10 mM inhibitor solution (Component E) 100-fold in 1X assay buffer (step 1.1) to get 100 µM diluted inhibitor solution. Add 10 µl of the diluted inhibitor solution into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and FAP enzyme solution (step 1.3) to the microplate wells. The suggested volume of enzyme solution for one well of a 96-well plate is 40 µL and test compound is 10 µL.

2.2 Establish the following control wells at the same time, as deemed necessary.

- Positive control: Add 40 µL FAP enzyme solution (step 1.3) and 10µL 1X assay buffer (step 1.1).
- Inhibitor control: Add 40 µL FAP enzyme solution (step 1.3) and 10µL FAP inhibitor (step 1.4).
- Vehicle control: Add 40 µL FAP enzyme solution (step 1.3) and 10µL vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control: Add 40µL 1X assay buffer (step 1.1) and 10µL test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control: Add 50µL 1X assay buffer (step 1.1).

2.3 The total volume of all controls should be 50 µL.

2.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

3. Detect FAP enzymatic activity.

3.1 Add 50 µL of the FAP substrate solutions (step 1.2) into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for no more than 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490 nm/520 nm, continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

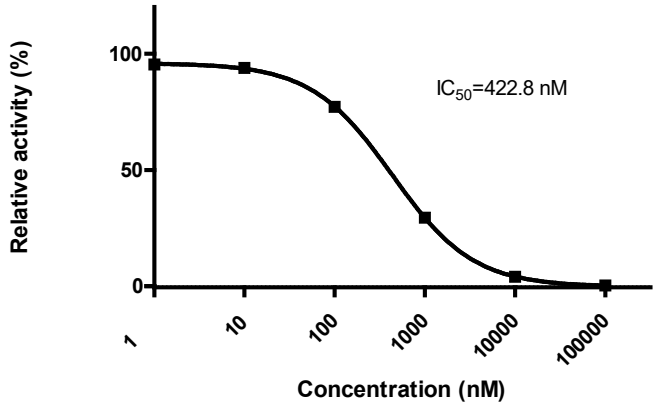

Figure 1. Inhibition of FAP activity by Talabostat mesylate measured with SensoLyte® 520 FAP Assay Kit. (SpectraMax M5 Microplate Reader, Molecular Devices)

3.3 For methods of data analysis: Refer to Appendix I.

Protocol B. Measuring FAP activity in biological samples

1. Prepare working solutions.

1.1.FAP substrate solution: Dilute FAP substrate (Component A) 1:100 in 2X assay buffer (Component D) according to Table 1. For each experiment prepare fresh substrate solution.

Table 1. FAP substrate solution for one 96-well plate (100 assays).

Components	Volume
FAP substrate (100X, Component A)	50 µL
2X Assay buffer (Component D)	4.95 mL
Total volume	5.0 mL

1.2 FAP enzyme solution: If you use purified FAP enzyme as a positive control, then dilute the enzyme (Component C) 1:500 in 1X assay buffer (Refer to Protocol A, step 1.1 for preparation). The suggested volume of enzyme solution for positive control is 50 µL/well (96-well plate).

Note 1: Mix the enzyme solution gently. Vigorous vortexing will denature the enzyme. Keep the enzyme on ice before use.
Note 2: For positive control use substrate solution diluted in 1X assay buffer as described in Protocol A, step 1.2.

2. Set up the enzymatic reaction.

2.1 Add 50 µL of FAP containing biological sample.

Note: Tissue extracts and cell lysates can be prepared with assay buffer provided in the kit

2.1 Set up the following control wells at the same time, as deemed necessary:

- Positive control: Add 50µL FAP enzyme solution (step 1.2).
- Substrate control: Add 50µL deionized water.

2.3 The total volume of all controls should be 50 µL.

2.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

3. Detect FAP enzymatic activity.

3.1 Add 50 µL of FAP substrate solution (step1.1) into each well. For best accuracy, it is advisable to have substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for no more than 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490 nm/520 nm, continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

3.3 For methods of data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetic analysis:
  - Plot data as RFU versus time for each sample. To convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min by determining 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 analysis:
  - 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 (optional)

- Fluorescence reference standard: Dilute 0.4 mM HiLyte Fluor™488 (Component B) 100-fold to 4 µM with 1X assay buffer (Refer to Protocol A, step 1.1 for preparation). Do 2-fold serial dilutions to get concentrations of 2, 1, 0.5, 0.25, 0.125 and 0.063 µM, include assay buffer blank. Add 50 µL/well of these serially diluted HiLyte Fluor™488 reference solutions.
- Add 50 µL/well of the diluted FAP substrate solution (refer to the protocol A, step 1.2 for preparation).

  Note: FAP substrate solution is added to the HiLyte Fluor™488 reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence of the reference standard and substrate control wells at Ex/Em=490nm/520nm. Use the same setting of sensitivity and temperature as used in the enzyme reaction.
- Plot the HiLyte Fluor™488 fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of HiLyte Fluor™488 reference standard are 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, and 0 µM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

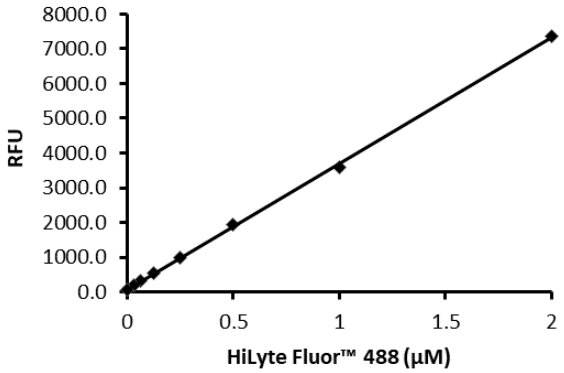

Figure 2. HiLyte Fluor™488 reference standard. HiLyte Fluor™488 was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm. (SpectraMax M5 Microplate Reader, Molecular Devices).

References

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