



SensoLyte® 520 β -Secretase Assay Kit

Fluorimetric

Revision number: 1.2

Last updated: October 2014

Catalog #	AS-71144
Kit Size	100 Assays (96-well plate)

- **Optimized Performance:** This kit is optimized to detect β -secretase activity.
- **Enhanced Value:** It provides enough reagents to perform 100 assays in a 96-well 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	β -secretase substrate	60 μ L
Component B	HiLyte Fluor™ 488, fluorescence reference standard	1 mM DMSO solution, 20 μ L
Component C	β -secretase Inhibitor	250 μ M, 10 μ L
Component D	2X Assay buffer	20 mL
Component E	Stop Solution	10 mL
Component F	Human β -secretase	0.5 mg/mL, 20 μ L

Other Materials Required (but not provided)

- 96-well microplate: Black microplates provide better signal to noise ratio.
- Fluorescence microplate reader: Capable of detecting excitation at 490 nm and emission at 520 nm.

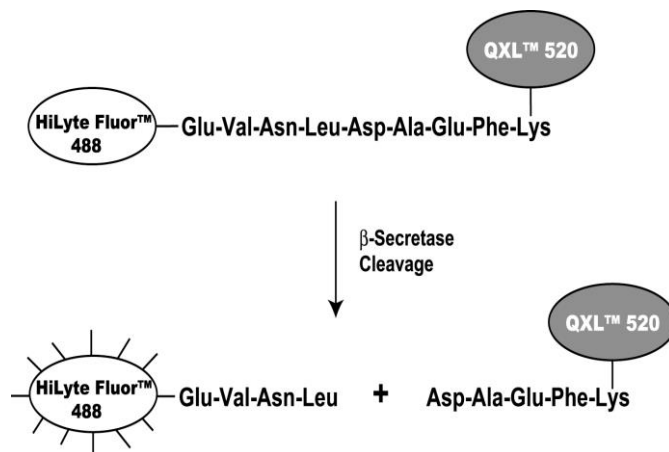
Storage and Handling

- Store all kit components at -20°C, except for Component F.
- Store β -secretase (Component F) at -80°C.
- Protect Components A and B from light and from moisture.
- Components D and E can be stored at room temperature for convenience.

Introduction

β -secretase is a membrane-anchored aspartic protease existing in acidic subcellular vesicles. It is a key player in producing the β -amyloid peptide through proteolytic cleavage of the β -amyloid precursor protein (APP). β -amyloid peptide is a neurotoxic peptide accumulated in senile plaques in the brains of patients with Alzheimer's disease (AD), an age-related cognitive disorder¹⁻⁴. Thus, β -secretase is an important target for developing drugs for AD.

The SensoLyte[®] β -Secretase Assay Kit provides a convenient assay for high throughput screening of β -secretase inhibitors and continuous quantification of β -secretase activity using a fluorescence resonance energy transfer (FRET) peptide, HiLyte Fluor[™] 488-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys (QXL[™] 520)-OH. The sequence of this FRET peptide is derived from the β -secretase cleavage site on the Swedish APP mutation⁵. This mutation enhances β -secretase to process APP and results in an early onset of AD. In the FRET peptide, the fluorescence of HiLyte Fluor[™] 488 is quenched by QXL[™] 520 until this peptide is cleaved into two separate fragments by β -secretase at the Leu-Asp bond. Upon cleavage, the fluorescence of HiLyte Fluor[™] 488 is recovered, and can be continuously monitored at excitation/emission = 490 nm/520 nm (Scheme 1). The assays are performed in a convenient 96-well microplate format. 384-well or 1536-well format can be used as well with minor modifications.



Scheme 1: The proteolytic cleavage of FRET peptide by β -secretase

Protocol

Note 1: For standard curve, please refer to [Appendix II](#) (optional).

Note 2: Please use protocol A or B based on your needs.

Note 3: Keep β -secretase on ice before use. Warm up the rest of the 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 β -secretase inhibitors using purified enzyme

1. Prepare working solutions.

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

1.2 β -secretase substrate solution: Dilute β -secretase substrate (Component A) 1:100 in 1X assay buffer according to Table 1. For each experiment prepare fresh substrate solution.

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

Components	Volume
β -secretase substrate (100X, Component A)	50 μ L
1X Assay buffer	4.95 mL
Total volume	5 mL

1.3 β -secretase diluent: Dilute the enzyme (Component F) 1:200 in 1X assay buffer. 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 diluents immediately before use. Do not vortex the enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 β -secretase inhibitor (LY2886721): Dilute 250 μ M inhibitor solution (Component C) 100-fold to 2.5 μ M in 1X assay buffer. Add 10 μ L of the 2.5 μ M inhibitor solution into each of inhibitor control well of a 96-well plate.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. The suggested volume of enzyme solution for a 96-well plate is 40 μ L/well and test compound is 10 μ L/well.

2.2 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains the enzyme and inhibitor.
- Vehicle control contains β -secretase enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains 1X assay buffer and test compound.
- Substrate control contains 1X assay buffer

2.3 Using the 1X assay buffer bring the total volume of all controls to 50 μ L.

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. Run the enzymatic reaction

3.1 Add 50 μ L of β -secretase substrate solution into each well. For best accuracy, it is advisable to have the β -secretase substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

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 min. for 30 min.

- For end-point reading: Incubate the reaction for 30 min. Keep plate from direct light. Optional: Add 50 μL of stop solution (Component E) to each well. Mix the reagents and measure fluorescence intensity at $\text{Ex/Em}=490\text{ nm}/520\text{ nm}$.

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

Protocol B. Measuring β -secretase activity in biological samples.

1. Prepare working solutions.

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

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

Components	Volume
β -secretase substrate (100X, Component A)	50 μL
2X Assay buffer (Component D)	4.95 mL
Total volume	5 mL

1.2 β -secretase diluent: If you use purified β -secretase enzyme as a positive control, then dilute the enzyme (Component F) 1:250 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 diluent gently. Vigorous vortexing will denature the enzyme. Keep the enzyme on ice before use.

Note 2: For positive control use secretase 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 β -secretase containing biological sample.

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

- Positive control contains purified active β -secretase
- Substrate control contains deionized water

2.3 Adjust the total volume of all controls to 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. Run the enzymatic reaction

3.1 Add 50 μL of β -secretase substrate solution into each well. For best accuracy, it is advisable to have the β -secretase substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at $\text{Ex/Em}=490\text{ nm}/520\text{ nm}$ continuously and record data every 5 min. for 30 min.
- For end-point reading: Incubate the reaction for 30 min. Keep plate from direct light. Optional: Add 50 μL of stop solution (Component E) to each well. Mix the reagents and measure fluorescence intensity at $\text{Ex/Em}=490\text{ nm}/520\text{ nm}$.

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 kinetics analysis:
 - Plot data as RFU versus time for each sample. If you want 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_0) 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

- HiLyte FluorTM 488 fluorescence reference standard: Dilute 1 mM HiLyte FluorTM 488 (Component B) 1:100 to 10 μM in 1X assay buffer. Do 1:2 serial dilutions to get

concentrations of 5, 2.5, 1.25, 0.625, 0.31, and 0.15 μM , include an assay buffer blank. Add 50 μL /well of these serially diluted HiLyte Fluor™ 488 reference solutions.

- Add 50 μL /well of the diluted β -secretase substrate solution (refer to the protocol for preparation).

Note: The β -secretase 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.

- Plot the HiLyte Fluor™ 488 fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 1.
- The final concentrations of HiLyte Fluor™ 488 reference standard are 5, 2.5, 1.25, 0.625, 0.31, 0.15, 0.07, 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.

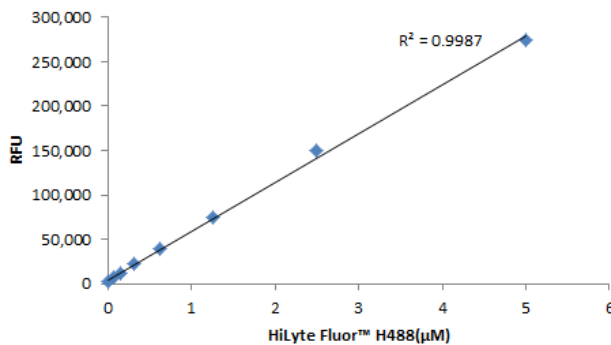


Figure 1. Standard curve of HiLyte Fluor™ 488. Fluorescent signal of serially diluted HiLyte Fluor™ 488 was monitored at Ex/Em=490 nm/ 520 nm (Flexstation 384 II, Molecular Devices).

REFERENCES

1. Vassar, R. et al. *Science* **286**, 735 (1999).
2. Glenner, GG. et al. *Biochem. Biophys. Res. Commun.* **120**, 885 (1984).
3. Selkoe, DJ. *Nature* **399**, A23 (1999).
4. Sinha, S. et al. *Proc. Natl. Acad. Sci. U.S.A* **96**, 11049 (1999).
5. Mullan, M. et al. *Nat. Genet.* **1**, 345 (1992).
6. Sinha, S. et al. *Nature* **402**, 537 (1999).