SensoLyte® 520 BACE2 (β-Secretase-2) Activity Assay Kit *Fluorimetric*

Catalog #	72225
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect BACE2 enzyme activity.
- ***Enhanced Value:*** 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 BACE2 substrate, Ex/Em=490/520 nm upon cleavage	1 mM, 50 µL
Component B	HiLyte Fluor™488, fluorescence reference standard, Ex/Em=490/520 nm	1 mM, 15 µL
Component C	Assay Buffer	30 mL
Component D	BACE2 Inhibitor	50 µM, 15 µL
Component E	Stop Solution	10 ml

Other Materials Required (but not provided)

- BACE2 source: Active enzyme (R&D Systems, Cat# 4097-AS-020)
- 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.
- Protect Components A, B, and D from light and moisture.
- Component C can be stored at 4 °C for convenience.

Introduction

BACE2 (β-Secretase-2, Memapsin-1) belongs to the family of transmembrane aspartic proteases.[1-5] BACE2 has approximately 75% homology with BACE1 (β-Secretase-1) and similar to that enzyme cleaves Amyloid Precursor Protein (APP) at β-site generating N-terminal part of beta-Amyloid peptide[1-5]. Unlike BACE1 that is highly expressed in pancreas and brain tissue, BACE2 is more widespread and can be found in many peripheral tissues at higher levels compared to BACE1.[1,5] BACE2 is one of the highest efficiency beta-amyloid peptide degrading enzyme that is less active only to insulin degrading enzyme (IDE); therefore, targeting BACE2 is considered a potential therapeutic strategy for the prevention and treatment of Alzheimer's disease.[4]

The SensoLyte® 520 BACE2 Assay Kit is a homogeneous assay that can be used to detect the activity of enzyme and for screening of BACE2 inhibitors. It contains a QXL™520/ HiLyte Fluor™488 FRET substrate, derived from a peptide sequence surrounding cleavage site of BACE2. QXL™520 quenches HiLyte Fluor™488 fluorescence in the intact FRET peptide. Active BACE2 cleaves FRET substrate into two separate fragments resulting in the release of HiLyte Fluor™488 fluorescence that can be monitored at excitation /emission= 490/520 nm. The long wavelength fluorescence of HiLyte Fluor™488 is less interfered by the autofluorescence of components in biological samples and test compounds.

Protocol

Note 1: To prepare a standard curve, please refer to Appendix II (optional).
Note 2: Please use Protocol A or B based on your needs.

Protocol A. BACE2 inhibitors screening with purified enzyme.

1. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

1.1 BACE2 substrate solution: Dilute BACE2 substrate (Component A) 1:100 in the assay buffer (Component C). For each experiment prepare fresh substrate solution.

Table 1. BACE2 substrate solution for one 96-well plate (100 assays)

Components	Volume
BACE2 Substrate (Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

1.2 BACE2 diluent: Dilute BACE2 enzyme to an appropriate concentration in the assay buffer (Component C).

Note: ***Recommended amount of enzyme is 100 ng/well.*** Prepare enzyme diluents immediately before use. Do not vortex enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store enzyme solution on ice.

1.3 BACE2 inhibitor (β-Secretase inhibitor IV): Dilute 50 µM inhibitor solution (Component E) 10-fold in 1X assay buffer to get 5 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 diluted enzyme solution 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 Simultaneously set up the following control wells, as deemed necessary:

- Positive control contains diluted BACE2 without test compound.
- Inhibitor control contains diluted BACE2 and supplied inhibitor (Step 1.3).
- Vehicle control contains BACE2 enzyme and vehicle used in delivering test compound (for example DMSO, concentration not to exceed 1%).
- 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 only.

2.3 Using the assay buffer bring total volume of all controls to 50 µL.

2.4 Optional: Cover plate and pre-incubate it for 10 minutes at the assay temperature. Any temperature (*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 the BACE2 substrate solution (Step 1.1) into each well. For best accuracy, equilibrate substrate solution to the assay temperature. Mix 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 minutes for 30 to 60 minutes.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light.

  Optional: Add 50 µl of Stop Solution (Component E) into each well. Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

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

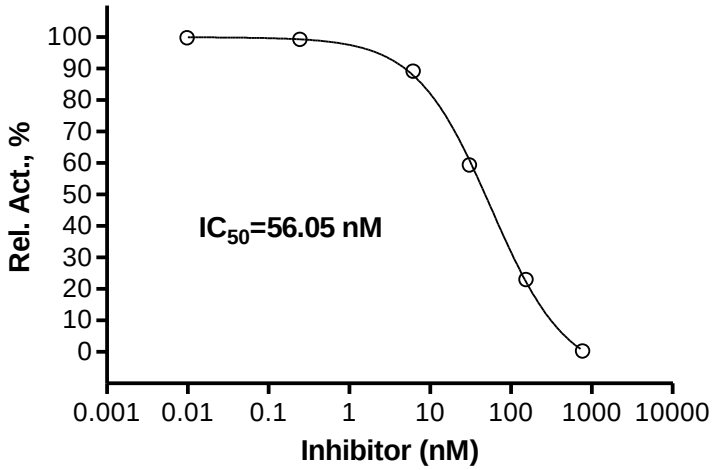

Figure 1. Inhibition of BACE2 activity by β-secretase inhibitor IV as measured with SensoLyte® 520 BACE2 Assay Kit.

Protocol B. Measuring BACE2 activity in biological samples.

1. Prepare BACE2 containing biological samples.

1.1 Prepare cell extract samples:

- Collect and wash cell with phosphate buffered saline (PBS).
- Add an appropriate amount of lysis buffer to cells or cell pellet. Collect cell suspension in a microcentrifuge tube. It is optional to use Assay Buffer (Component C) containing 0.1% (v/v) Triton X-100.
- Incubate cell suspension at 4 °C for 10-15 minutes.
- Centrifuge mixture for 10 minutes at 2,500 x g at 4 °C. Collect supernatant and store it at -70°C until use.

1.2 Prepare tissue extract samples:

- Collect tissue.
- Homogenize tissue samples in the lysis buffer. It is optional to use Assay Buffer (Component C) containing 0.1% (v/v) Triton X-100.
- Incubate for 10 min on ice.
- Centrifuge mixture for 15 min. at 14,000 x g, 4 °C. Collect supernatant and store it at -70°C until use.

Note 1: PBS is not provided. Cell or tissue extract should be diluted and used as the enzyme source to measure BACE2 activity.

2. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

2.1 BACE2 substrate solution: Dilute BACE2 substrate (Component A) 1:100 in the assay buffer (Component C). For each experiment prepare fresh substrate solution.

Table 1. BACE2 substrate solution for one 96-well plate (100 assays)

Components	Volume
BACE2 Substrate (Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

2.2 BACE2 diluent: If purified BACE2 is used as a positive control, it must be diluted to an appropriate concentration in the assay buffer (Component C).

Note: Prepare enzyme diluents immediately before use. Do not vortex enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store enzyme solution on ice.

3. Set up the enzymatic reaction.

3.1 Add 50 µL of BACE2 containing sample.

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

- Positive control contains purified active BACE2.
- Substrate control contains assay buffer.

Note: If measuring BACE2 activity on live cells, establish additional controls: baseline control (contains cells incubated without substrate) and cell medium control (contains cell medium only)

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

3.4 Optional: Pre-incubate the plate for 10 minutes at the 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.

4. Run the enzymatic reaction.

4.1 Add 50 µL of the BACE2 substrate solutions into each well. For best accuracy, equilibrate substrate solution to the assay temperature. Mix reagents completely by shaking plate gently for 30 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence 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 for 30 to 60 minutes. Keep plate from direct light. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

  Optional: Add 50 µl of Stop Solution (Component E) into each well. Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 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. 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 range of initial time points during which the reaction is linear. Typically, first 10-15% of the reaction will be the optimal range.
  - Obtain initial reaction velocity (V_o) in RFU/min by determining slope of the linear portion of 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

- Fluorescence reference standard: Dilute 1 mM fluorescence standard solution (Component B) 100-fold to 10 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 5, 2.5, 1.25, 0.63, 0.32, and 0.16 μM, include assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.
- Add 50 μL/well of the diluted BACE2 substrate solution (refer to Protocol A, Step 1.2 for preparation).

  Note: BACE2 substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure fluorescence of the reference standard and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzymatic reaction.
- Plot reference standard curve as RFU (relative fluorescent units) versus concentration.
- The final concentrations of fluorescence reference standard are 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, and 0 μM. This reference standard is used to calibrate variations of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

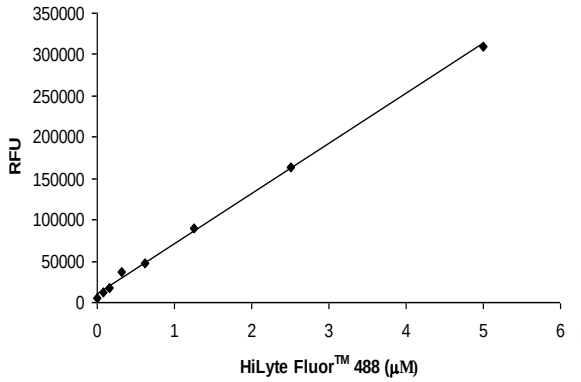

Figure 3. HiLyte Fluor™488 reference standard. HiLyte Fluor™488 standard solution was serially diluted in the assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References :

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