SensoLyte® 520 Neprilysin Activity Assay Kit *Fluorimetric*

Catalog #	72223
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect neprilysin 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	5-FAM/QXL™-520 Neprilysin substrate, Ex/Em=490/520 nm upon cleavage	1 mM, 50 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490/520 nm	1 mM, 15 µL
Component C	Recombinant human neprilysin	10 µg/mL, 100 µL
Component D	2X Assay Buffer	30 mL
Component E	Inhibitor	0.1 mM, 15 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.
- Protect Components A and B from light and moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Neprilysin (NEP) is a transmembrane metallopeptidase normally expressed by a variety of tissues.[1,2] It is also known as neutral endopeptidase, and enkephalinase**.** NEP cleaves peptides at the N-terminal side of hydrophobic amino acid residues and is responsible for the degradation and inactivation of a variety of physiological substrates.[3] NEP is a major extracellular amyloid beta-peptide degrading enzyme in the brain and targeting NEP is considered a potential therapeutic strategy for the prevention and treatment of Alzheimer's disease.[4,5] NEP has also been implicated in the pathogenesis of hypertension, diabetes, and cancer.[6-8]

The SensoLyte® 520 Neprilysin Assay Kit employs a novel internally quenched 5-FAM/QXL™ FRET substrate for the detection of neprilysin activity. The enzyme cleaves the FRET substrate into two separate fragments resulting in the release of 5-FAM fluorescence, which can be monitored at excitation /emission= 490/520 nm. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of components in biological samples and test compounds.

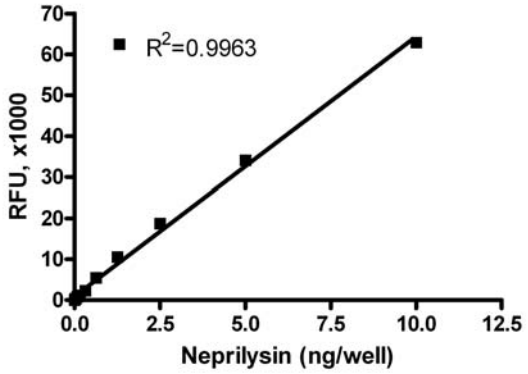

Figure 1. Sensitivity of the assay has been tested using serial dilutions of recombinant human neprilysin. 5-FAM/QXL™ 520 FRET substrate was incubated with the indicated amount of enzyme and fluorescence was measured after 60 min (FlexStation 384II, Molecular Devices). The assay can detect as low as 0.78 ng/mL of active neprilysin.

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. Screening compounds using purified enzyme.

1. Prepare working solutions.

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

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

1.2 Neprilysin substrate solution: Dilute neprilysin substrate (Component A) 100-fold in 1X assay buffer. Refer to Table 1. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

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

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

1.3 Neprilysin diluents: Dilute neprilysin enzyme (Component C) 40-fold 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 solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 Neprilysin inhibitor (Thiorphan): Dilute the 0.1 mM inhibitor solution (Component E) 100-fold in 1X assay buffer to get 1.0 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 the diluted neprilysin without test compound.
- Inhibitor control contains the diluted neprilysin and inhibitor.
- Vehicle control contains 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. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains 1X assay buffer.

2.3 Using the 1X assay buffer, bring 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 the neprilysin substrate solutions 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 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. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

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

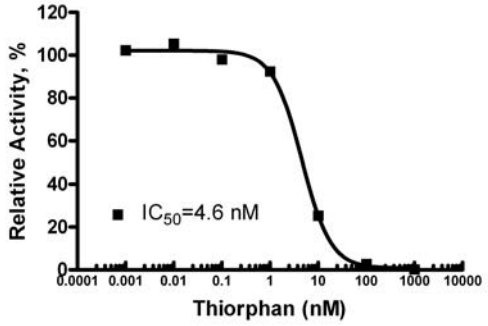

Figure 2. Inhibition of neprilysin activity by Thiorphan as measured with SensoLyte® 520 Neprilysin Assay Kit.

Protocol B. Measuring neprilysin activity in biological samples.

1. Prepare neprilysin containing biological samples.

1.1 Prepare cell extract samples:

- Collect cells and wash cell pellets with phosphate buffered saline (PBS).
- Lyse cells, and centrifuge at 15,000x g for 5 min, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare tissue extract samples:

- Collect tissues.
- Homogenize tissue samples, and centrifuge at 15,000x g for 5 min at 4°C.
- Collect the supernatant and store 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 neprilysin activity.

Note 2: It is optional to use our assay buffer (1X) for preparation of biological samples. If using our assay buffer, the neprilysin substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer.

2. Prepare working solutions.

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

2.1 The neprilysin substrate solution: Dilute neprilysin substrate (Component A) 100-fold in 2X assay buffer (Component D). Refer to Table 1. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

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

Components	Volume
Neprilysin substrate (Component A)	50 µL
2X assay buffer (Component D)	4.95 mL
Total volume	5 mL

3. Set up the enzymatic reaction.

3.1 Add 50 µL of neprilysin containing sample.

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

- Positive control contains purified active neprilysin.

- Substrate control contains deionized water.

3.3 Bring the total volume of all controls to 50 µL.

3.4 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.

4. Run the enzymatic reaction.

4.1 Add 50 µL of the neprilysin substrate solutions 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 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 min for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Mix the reagents and 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 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

- Fluorescence reference standard: Dilute the 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 an assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.
- Add 50 μL/well of the diluted neprilysin substrate solution (refer to Protocol A, Step 1.2 for preparation).

  Note: The neprilysin 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 the 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 enzyme reaction.
- Plot the 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 the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

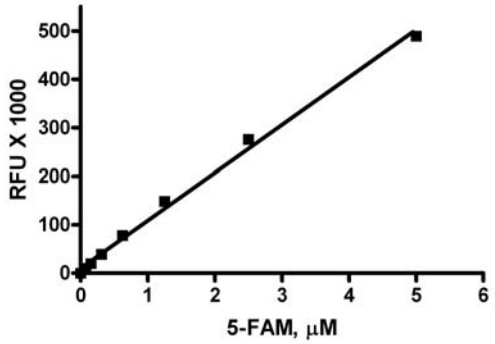

Figure 3. 5-FAM reference standard. 5-FAM standard solution was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References

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