Neprilysin Activity Assay Kit (Fluorimetric)

Cat. No. KITG-149 **Lot. No.** (See product label)

SPECIFICATION

Product Overview	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: a detailed protocol is provided.
Description	Neprilysin (NEP) is a transmembrane metallopeptidase normally expressed by a variety of tissues. 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. 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. NEP has also been implicated in the pathogenesis of hypertension, diabetes, and cancer. This assay kit employs a novel internally quenched 5-FAM 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.
Form	Size 100 Assays (96-well plate) Kit Components Component A: neprilysin substrate (internally quenched 5-FAM FRET substrate), Ex/Em = 490/520 nm upon cleavage; 1 mM, µL x 1 vial (50 µL). Component B: 5-FAM fluorescence reference standard, Ex/Em = 490/520 nm; 1 mM, µL x 1 vial (15 µL). Component C: recombinant human neprilysin; 10 µg/mL, µL x 1 vial (100 µL). Component D: 2X assay buffer; µL x 1

Tel: 1-631-559-9269 1-516-512-3133
Email: info@creative-biomart.com Fax: 1-631-938-8127
45-1 Ramsey Road, Shirley, NY 11967, USA

	bottle (30 mL). Component E: inhibitor (thiorphan); 0.1 mM, µL x 1 vial (15 µL).
Bio-activity	Sensitivity The assay can detect as low as 0.78 ng/mL of active neprilysin. Sensitivity was tested using serial dilutions of recombinant human neprilysin incubated with the 5-FAM FRET substrate, with fluorescence measured after 60 min.
Applications	Applications Screening of test compounds using purified enzyme (Protocol A); measurement of neprilysin activity in biological samples (Protocol B). Detection Method Fluorometric assay. Measuring instrument: fluorescence microplate reader capable of detecting emission at 520 nm with excitation at 490 nm. Compatible Sample Types Purified enzyme preparations; cell extracts; tissue extracts.
Notes	Notes 1. Bring all kit components until thawed to room temperature before starting the experiments. 2. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly. 3. Prepare the enzyme dilution 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. 4. 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. 5. Some test compounds have strong autofluorescence and may give false results; a test compound control is therefore recommended. The vehicle used to deliver the test compound (e.g. DMSO) should not exceed 1%. 6. Cell or tissue extract should be diluted and used as the enzyme source to measure neprilysin activity. PBS is not provided. It is optional to use the 1X assay buffer for the preparation of biological samples; if the assay buffer is used, the neprilysin substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer. 7. Collected cell or tissue extract supernatants can be stored at -70°C until use. 8. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature before it is added to the wells. Technical Notes The neprilysin substrate solution is added to the fluorescence reference standard to

	normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration. Use the same sensitivity setting of the instrument for the reference standard as used in the enzyme reaction.
Storage	Storage 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.
Reaction conditions	Preparation Note: bring all kit components until thawed to room temperature before starting the experiments. 1. 1X assay buffer: add 10 mL of 2X assay buffer (Component D) to 10 mL of deionized water. 2. Neprilysin substrate solution: dilute the neprilysin substrate (Component A) 100-fold in 1X assay buffer (Protocol A) or in 2X assay buffer, Component D (Protocol B). 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): neprilysin substrate (Component A) 50 µL; 1X assay buffer (Protocol A) or 2X assay buffer, Component D (Protocol B) 4.95 mL; total volume 5 mL. Note: in Protocol B, if the 1X assay buffer is used for the preparation of biological samples, the neprilysin substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer. 3. Neprilysin dilution (Protocol A): dilute the 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. Prepare the enzyme dilution 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. 4. Neprilysin inhibitor solution (Protocol A): dilute the 0.1 mM inhibitor solution (Component E) 100-fold in 1X assay buffer to obtain a 1.0 µM diluted inhibitor solution. Add 10 µL of the diluted inhibitor solution into each inhibitor control well. Assay Protocol Protocol A. Screening compounds using purified enzyme. 1. Add the test compounds and the

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 that of the test compound is 10 µL. 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 the inhibitor. • Vehicle control: contains the enzyme and the vehicle used in delivering the test compound (e.g. DMSO, concentration not to exceed 1%). • Test compound control: contains 1X assay buffer and the test compound. Some test compounds have strong autofluorescence and may give false results. • Substrate control: contains 1X assay buffer. 3. Using the 1X assay buffer, bring the total volume of all controls to 50 µL. 4. Optional: pre-incubate the plate for 10 min at the assay temperature. Any temperature from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature. 5. Add 50 µL of the neprilysin substrate solution 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. 6. Measure the 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 the plate from direct light, mix the reagents, and measure the fluorescence intensity at Ex/Em = 490 nm/520 nm. Protocol B. Measuring neprilysin activity in biological samples. 1. Prepare the neprilysin-containing biological samples. • Cell extract samples: collect the cells and wash the cell pellets with phosphate buffered saline (PBS). Lyse the cells and centrifuge at 15,000×g for 5 min at 4°C. Collect the supernatant and store it at -70°C until use. • Tissue extract samples: collect the tissues. Homogenize the tissue samples and centrifuge at 15,000×g for 5 min at 4°C. Collect the supernatant and store it at -70°C until use. Note: PBS is not provided. The cell or tissue extract should be diluted and used as the enzyme source to measure neprilysin activity. It is optional to use the 1X assay buffer for the preparation of biological samples; if the assay buffer is used, the

neprilysin substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer. 2. Prepare the working solutions. Bring all kit components until thawed to room temperature before starting the experiments. Prepare the neprilysin substrate solution as described above: dilute the neprilysin substrate (Component A) 100-fold in 2X assay buffer (Component D), referring to Table 1. 3. Set up the enzymatic reaction. 3.1 Add 50 µL of the neprilysin-containing sample. 3.2 Set up the following control wells at the same time, as deemed necessary: the positive control contains purified active neprilysin; the 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 the assay temperature. Any 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 solution 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 the 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 the plate from direct light, mix the reagents, and measure the fluorescence intensity at Ex/Em = 490 nm/520 nm. Assay time Optional pre-incubation: 10 min at the assay temperature. Enzymatic reaction: 30 to 60 min (kinetic reading: record data every 5 min for 30 to 60 min). The entire process can be completed in one hour. 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 the data as RFU versus time for each sample. To convert RFUs to the concentration of the product of the enzymatic reaction, establish a fluorescence reference standard curve as described below. 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 %, EC50, IC50, Km, Ki, etc. • For endpoint analysis: plot the data as RFU versus concentration of the test compounds. A variety of data analyses can be done, e.g., determining inhibition %, EC50, IC50, etc. Instrument Calibration (fluorescence reference standard, optional) 1. Dilute the 1 mM fluorescence standard solution (Component B) 100-fold to 10 µM in 1X assay buffer. Perform 2-fold serial dilutions to obtain concentrations of 5, 2.5, 1.25, 0.63, 0.32, and 0.16 µM, and include an assay buffer blank. Add 50 µL/well of these serially diluted reference solutions. 2. Add 50 µL/well of the diluted neprilysin substrate solution. 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. 3. Measure the fluorescence of the reference standard and substrate control wells at Ex/Em = 490 nm/520 nm. Use the same sensitivity setting as used in the enzyme reaction. 4. Plot the reference standard curve as RFU (relative fluorescence units) versus concentration. 5. The final concentrations of the 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 between different instruments and different experiments, and is also an indicator of the amount of final product of the enzymatic reaction.
Usage	Usage Protocol A is used for screening compounds with purified enzyme; Protocol B is used for measuring neprilysin activity in biological samples (cell extracts and tissue extracts). Cell or tissue extract should be diluted and used as the enzyme source. Materials Required but Not Supplied • 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. • Phosphate buffered saline (PBS): for washing cell pellets. • Deionized water.

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