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Neprilysin Activity Assay Kit (Fluorometric)

(Catalog # K487-100; 100 assays; Store at -20°C)

I. Introduction:

Neprilysin (NEP, EC 3.4.24.11), also known as neutral endopeptidase, enkephalinase, CD10, and common acute lymphoblastic leukemia antigen, is a zinc-containing transmembrane metalloproteinase. It is able to hydrolyze very important endogenous peptides, such as natriuretic atrial factor, enkephalins, substance P, bradykinin and amyloid β (Aβ) peptide. Thus, NEP is a potentially therapeutic target in important pathological conditions such as cardiovascular disease, prostate cancer, and Alzheimer's disease. NEP has also been used as a biological marker of a type of child leukemia and the detection of NEP in endometrial stromal cells had been proposed as a helpful tool in diagnosis of endometriosis. NEP is currently a focus of major interest in cardiovascular and neurological research. BioVision's Neprilysin Activity Kit utilizes the ability of an active NEP to cleave a synthetic substrate (Abz-based peptide) to release a free fluorophore. The released Abz can be easily quantified using a fluorescence microplate reader. The substrate is specific to NEP and can differentiate the NEP activity from Trypsin and other structurally similar zinc metalloproteinase in biological samples such as Angiotensin-Converting Enzymes (ACE1, ACE2), Endothelin Converting Enzymes (ECE1, ECE2). Our assay kit is simple, specific and can detect as low as 20 µU/mg of NEP activity.

NEP Substrate-Abz $\xrightarrow{\text{Neprilysin}}$ Cleaved Substrate + Abz-Fluorescent Product (Ex/Em= 330/430 nm)

II. Applications:

- Measurement of Neprilysin activity in various biological samples/preparations

III. Sample Type:

- Tissue homogenates: lung, kidney, etc.
- Cell culture: adherent or suspension cells
- Purified enzyme

IV. Kit Contents:

Components	K487-100	Cap Code	Part Number
NEP Assay Buffer	40 ml	NM	K487-100-1
Neprilysin (Lyophilized)	1 vial	Green	K487-100-2
NEP Substrate (in DMSO)	15 µl	Red	K487-100-3
Abz-Standard (1 mM)	100 µl	Yellow	K487-100-4

V. User Supplied Reagents and Equipment:

- 96-well white opaque plate
- Multi-well spectrophotometer (fluorescence plate reader)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protect from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **NEP Assay Buffer:** Store at either 4 ºC or -20 ºC. Bring to room temperature before use.
- **Neprilysin:** Reconstitute Neprilysin in 500 µl NEP Assay Buffer and mix thoroughly. Aliquot and store at -20°C. Avoid repeated freeze/thaw. Keep on ice while in use. Use within two months.
- **NEP Substrate and Abz-Standard:** Store at -20ºC, protect from light.

VII. Neprilysin Activity Assay Protocol:

1. **Sample Preparation:** Homogenize tissue (~100 mg) or pelleted cells (~1-2 X10^6) with 400 µl of iced-cold NEP Assay Buffer containing protease inhibitors (we suggest use 1 mM PMSF and 10 µg/ml Aprotinin) and keep on ice for 10 min. Centrifuge samples at 12,000 x g at 4 ºC for 10 min. and collect the supernatant. Add 1-10 µl (**see note b**) of sample into desired well(s) in a 96-well white plate labeled as Sample and Sample Background Control. For positive control, add 4-10 µl of Reconstituted Neprilysin into desired well(s). Adjust the volume of Positive Control, Sample Background Control and Sample wells to 90 µl/well with NEP Assay Buffer.

   Note:

   a. Neprilysin is zinc-containing transmembrane metalloproteinase. Tested samples should not contain EDTA/EGTA.

   b. Tissue or cell lysates of more than 15 µg of total protein/well might suppress the enzymatic activity of NEP with the provided substrate. For samples having high protein concentration, dilute the sample with NEP Assay Buffer and use 3-5 different amounts of the diluted samples per well to ensure the change of velocity of the readings is within the linear range.

   c. Some protease inhibitors might suppress the enzymatic activity of NEP with the provided substrate. We suggest use freshly prepared PMSF and Aprotinin.

   d. Equilibrate the NEP Assay Buffer to 37 ºC before adding to the wells.

2. **Standard Curve Preparation:** Prepare a 100 µM solution of Abz-Standard by diluting 10 µl of 1 mM Abz-Standard with 90 µl of NEP Assay Buffer. Add 0, 2, 4, 6, 8, and 10 µl of 100 µM Abz-Standard into a series of wells in a 96-well white plate and adjust the final volume to 100 µl/well with NEP Assay Buffer to generate 0, 200, 400, 600, 800 and 1000 pmol/well of Abz-Standard respectively, mix well.

   Note: Equilibrate the Standard Solution to 37 ºC before measuring.

3. **NEP Substrate Solution Preparation:** Prepare a 100-fold dilution of NEP Substrate Stock Solution (i.e. Dilute 2 µl of NEP Substrate with 198 µl of NEP Assay Buffer), vortex briefly. Add 10 µl of Diluted NEP Substrate Solution to each well containing Test Sample(s) and NEP positive control(s). For Sample Background Control, add 10 µl of NEP Assay Buffer. The total volume for each well is 100 µl.

   Note: Equilibrate the Substrate Solutions to 37 ºC before adding to the wells.

4. **Measurement:** Measure fluorescence (Ex/Em= 330/430nm) in kinetic mode at 37 ºC for 1-2 h. Standards (Step 2) can be read in endpoint mode. Choose two time points (t_1 and t_2) in the linear range of the plot and obtain the corresponding fluorescence values (RFU_1 and RFU_2).

   Note: Incubation time depends on the NEP activity in samples. Longer incubation time may be required for samples having low NEP activity.

5. **Calculation:** Subtract 0 Standard Reading from all Standard Readings. Plot the Abz Standard Curve and obtain the slope of the curve (**ΔRFU/pmol)**; apply Sample ΔRFU and Sample Background Control ΔRFU to Abz Standard Curve to obtain the corresponding amount of Abz formed. Calculate the background-corrected sample ΔRFU (B, in pmol) by subtracting the amount of Abz formed by Sample Background Control from the amount of Abz formed by Sample and calculate the activity of NEP activity in the sample as:

Sample NEP Activity = B/ (Δt X V) x D = pmol/min/ml = µU/ml

Where: **B** = Abz from Standard Curve (pmol)
Δt = Reaction time (min.)
V = Sample volume added into the reaction well (ml)
D = Sample Dilution Factor (D=1 when samples are undiluted)

Neprilysin specific activity can be expressed as U/mg of protein.

Unit Definition: One unit of NEP activity is the amount of enzyme that catalyzes the release of 1 µmol of Abz per min from the substrate under the assay conditions at 37 ºC.

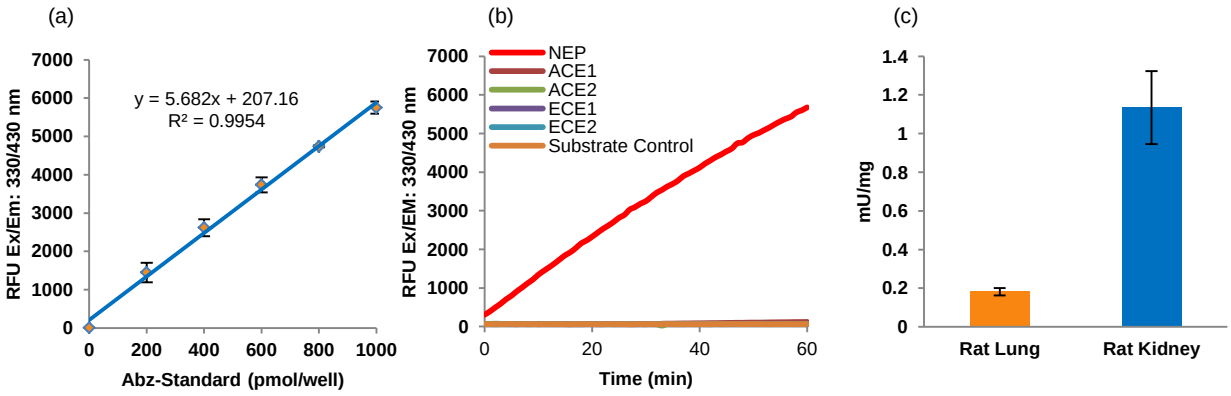

Figure: (a) Abz-Standard Curve, results from multiple experiments. (b) Measurement of purified NEP (10 ng), ACE1 (150 ng), ACE2 (0.5 ng), ECE1 (15 ng) and ECE2 (20 ng) activities using our proprietary substrate. The kit can efficiently distinguish NEP activity from other zinc metalloproteases in biological samples. (c) Measurement of NEP activity in Rat Lung (10 µg protein) and Rat Kidney (8.5 µg protein). All assays were performed following kit protocol.

VIII. RELATED PRODUCTS:

Human CellExp ™ Neprilysin/CD10, Human recombinant (7445-10)
Neprilysin Inhibitor Screening Kit (K996)
Angiotensin I converting enzyme (ACE1) Activity Assay Kit (K227)
Angiotensin I converting enzyme (ACE1) Inhibitor Screening Kit (K228)
Angiotensin II converting enzyme (ACE2) Activity Assay Kit (K897)
Angiotensin II converting enzyme (ACE2) Inhibitor Screening Kit (K310)
Renin Activity Fluorometric Assay Kit (K800)
Renin Inhibitor Screening Kit (Fluorometric) (K799)

FOR RESEARCH USE ONLY! Not to be used on humans.