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Product Information

Neprilysin Activity Assay Kit

Catalog Number **MAK350**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Neprilysin, 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 (A β) 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 in a type of childhood leukemia. The detection of NEP in endometrial stromal cells has been proposed as a helpful tool in the diagnosis of endometriosis. NEP is currently a focus of major interest in cardiovascular and neurological research.

The Neprilysin Activity Assay Kit utilizes the ability of an active NEP to cleave a synthetic substrate, *o*-amino-benzoic acid (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 and ACE2) and endothelin converting enzymes (ECE1 and ECE2). The assay kit is simple, specific, and can detect as low as 20 μU/mg of NEP activity. This kit is suitable for the measurement of neprilysin activity in tissue homogenates (lung, kidney, etc.), cell culture (adherent or suspension cells), and purified enzyme.

Components

The kit is sufficient for 100 fluorometric assays in 96 well plates.

NEP Assay Buffer Catalog Number MAK350A	40 mL
Neprilysin (Lyophilized) Catalog Number MAK350B	1 vial
NEP Substrate (in DMSO) Catalog Number MAK350C	15 μL
Abz-Standard (1 mM) Catalog Number MAK350D	100 μL

Reagents and Equipment Required but Not Provided.

- Pipetting devices and accessories (e.g., multichannel pipettor)
- White opaque flatbottom 96 well plates
- Fluorescence multiwell plate reader, capable of 37 °C temperature setting
- Refrigerated microcentrifuge capable of RCF ≥12,000 × *g*
- Protease Inhibitor Cocktail (contains 80 μM Aprotinin) (Catalog Number P8340)
- 0.1 M PMSF (Catalog Number 93482)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at –20 °C, protected from light upon receiving. Briefly centrifuge small vials prior to opening.

Preparation Instructions.

NEP Assay Buffer: Bring to room temperature before use.

Neprilysin: Reconstitute Neprilysin in 500 µL of NEP Assay Buffer and mix thoroughly. Aliquot and store at –20 °C. Avoid repeated freeze/thaw cycles. Keep on ice while in use. Stable for two months once reconstituted.

NEP Substrate Working Solution: Prepare a 100-fold dilution of NEP Substrate (i.e. Dilute 2 µL of NEP Substrate with 198 µL of NEP Assay Buffer), vortex briefly.

NEP Assay Buffer containing protease inhibitors:

Prepare 400 µL per sample just prior to use. Add protease inhibitors at the below concentrations to NEP Assay Buffer:

Aprotinin: 10 µg/mL
PMSF: 1 mM

Chill on ice until ready to use in assay.

Note: Some protease inhibitors might suppress the enzymatic activity of NEP with the provided substrate.

Sample Preparation

1. Homogenize tissue (~100 mg) or pelleted cells (1-2 x 10^6) with 400 µL of iced-cold NEP Assay Buffer containing protease inhibitors.
2. Keep on ice for 10 minutes.
3. Centrifuge samples at 12,000 × *g* at 4 °C for 10 minutes and collect the supernatant.
4. Add 1-10 µL (see note) of sample into desired well(s) in a 96 well white plate labeled as Sample and Sample Background Control.
   Note: 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.
5. For positive control, add 4-10 µL of Reconstituted Neprilysin into desired well(s).
6. Adjust the volume of Positive Control, Sample Background Control, and Sample wells to 90 µL/well with NEP Assay Buffer equilibrated to 37 °C.
   Note: Neprilysin is zinc-containing transmembrane metalloproteinase. Tested samples should not contain EDTA/EGTA.

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, mix well. Prepare Abz Standards according to Table 1. Mix well.

Table 1.
Preparation of Abz Standards

Well	100 µM Premix	NEP Assay Buffer	Abz (pmol/well)
1	0 µL	100 µL	0
2	2 µL	98 µL	200
3	4 µL	96 µL	400
4	6 µL	94 µL	600
5	8 µL	92 µL	800
6	10 µL	90 µL	1,000

Equilibrate the Standards to 37 °C before measuring.

Procedure

Assay Reaction

1. Equilibrate the NEP Substrate Working Solution to 37 °C.
2. Add 10 µL of NEP Substrate Working Solution to each well containing Test Sample(s) and NEP positive control(s).
3. For Sample Background Control, add 10 µL of NEP Assay Buffer.
4. The total volume for each well is 100 µL.

Measurement

1. Measure fluorescence (λ_{ex} = 330 nm/λ_{em} = 430 nm) in kinetic mode at 37 °C for 1-2 hours (incubation time depends on the NEP activity in the sample(s). Longer incubation time may be required for samples having low NEP activity).
2. Standards can be read in endpoint mode.
3. 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).

Results

1. Subtract 0 Standard Reading from all remaining Standard Readings.
2. Plot the Abz Standard Curve and obtain the slope of the curve (ΔRFU/pmol). Compare Sample ΔRFU and Sample Background Control ΔRFU to Abz Standard Curve to obtain the corresponding amount of Abz formed.
3. 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 (pmol/min/mL or μU/mL) =

$$B / (\Delta t \times V) \times D$$

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

Unit Definition

One unit of NEP activity is the amount of enzyme that catalyzes the release of 1 μmol of Abz per minute from the substrate under the assay conditions at 37 °C. Neprilysin specific activity can be expressed as U/mg of protein.

Figure 1.
Typical Abz-Standard Curve, results from multiple experiments.

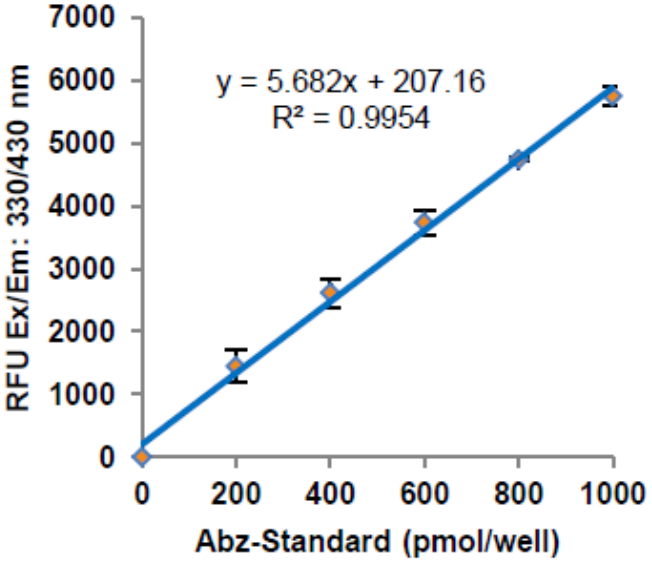


Figure 2.
Measurement of purified NEP (10 ng), ACE1 (150 ng), ACE2 (0.5 ng), ECE1 (15 ng), and ECE2 (20 ng) activities using the kit's proprietary substrate. The kit can efficiently distinguish NEP activity from other zinc metalloproteases in biological samples.

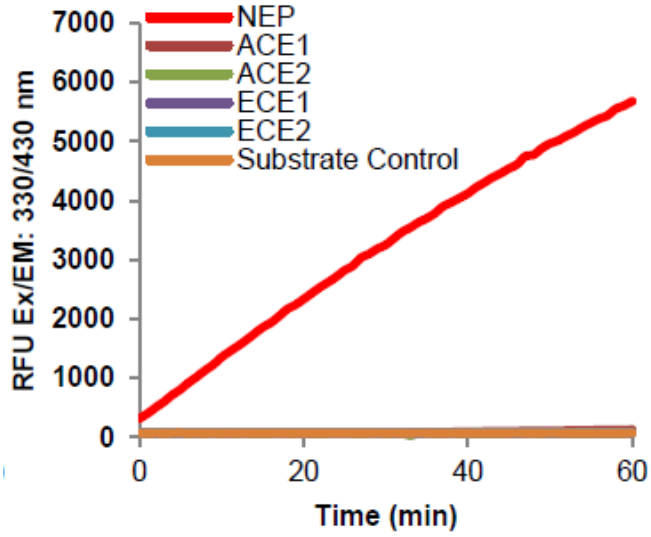
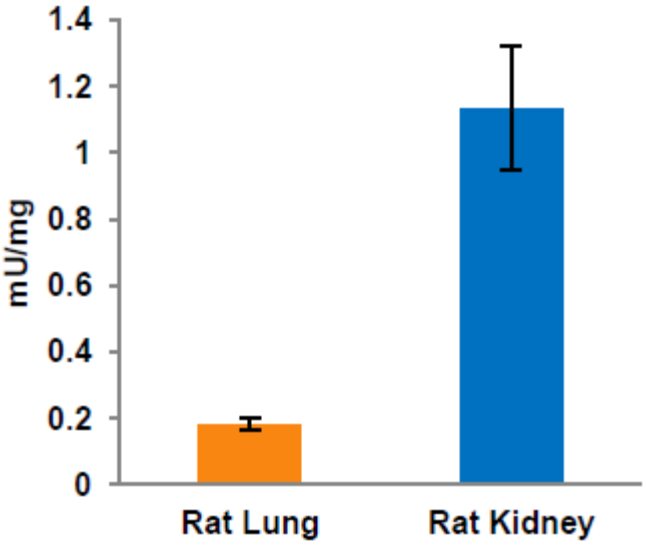


Figure 3.
Measurement of NEP activity in Rat Lung (10 μg protein) and Rat Kidney (8.5 μg protein). All assays were performed following the kit procedure.



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