Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

Endothelin Converting Enzyme 1 Activity Assay Kit (Fluorometric)

12/17

(Catalog # K536-100; 100 assays; Store at -20°C)

I. Introduction:

Endothelin Converting Enzyme 1 (ECE1; EC: 3.4.24.71) is a membrane-bound metalloprotease that cleaves inactive big endothelin (big ET-1), which is the precursor of active endothelin. Studies have shown that big ET-1 could be used as a predictor of survival in patients suffering esophageal squamous cell carcinoma. ECE-1 is abundantly expressed *in vivo* in endothelial cells producing mature ET-1. As of today, Endothelin is the most potent vasoconstrictor known and it has cytokine- or hormone-like activities. Therefore, ECE-1 could play a significant role in pathogenesis of cardiovascular diseases and Alzheimer's Disease. BioVision's Endothelin Converting Enzyme 1 Activity Assay Kit utilizes the ability of active ECE-1 to cleave a synthetic substrate (MCA-based peptide) releasing free fluorophore, in the presence or absence of the ECE-1 Inhibitor Mix. The released fluorophore can be easily quantified using a fluorescence microplate reader. Our kit uses a unique combination of substrate and inhibitor that specifically detects ECE-1 in a variety of biological samples. Contribution of other enzymes with similar catalytic properties - *i.e.* Endothelin Converting Enzyme 2 (ECE-2), Angiotensin-Converting Enzyme (ACE1, ACE2), and Neprilysin is compensated for within the assay. Our assay kit is simple, specific and can detect as low as 0.5 µU of ECE-1 activity.

ECE-1 Substrate-MCA —(ECE-1)→ Cleaved Substrate + MCA (Ex/Em= 320/420 nm)

ECE-1 Substrate-MCA —(ECE-1+ Inhibitor)→ Cleaved Substrate + MCA (Ex/Em= 320/420 nm) **(Low RFU)**

II. Applications:

- Measurement of ECE-1 activity in various biological samples/protein preparations

III. Sample Type:

- Tissue homogenates: lung, heart, etc.
- Cell culture: Hela Cell Lysates
- Purified enzyme/Protein Preparations

IV. Kit Contents:

Components	K536-100	Cap Code	Part Number
ECE-1 Assay Buffer	50 ml	NM	K536-100-1
ECE-1 (lyophilized)	1 vial	Green	K536-100-2
ECE-1 Substrate (in DMSO)	55 µl	Red	K536-100-3
ECE-1 Inhibitor Mix	1 vial	Blue	K536-100-4
MCA Standard (5 mM)	40 µl	Yellow	K536-100-5

V. User Supplied Reagents and Equipment:

- 96-well white opaque plate
- Multi-well spectrophotometer (fluorescence plate reader)
- Dounce Tissue Homogenizer (Cat. #1998 or equivalent)

VI. Storage Conditions and Reagent Preparation:

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

- **ECE-1 Assay Buffer:** Warm to 37 ºC before use. Store at either 4 ºC or -20 ºC.
- **ECE-1:** Reconstitute ECE-1 in 60 µl ECE-1 Assay Buffer and mix thoroughly. Aliquot and store at **-80 °C**. Avoid repeated freeze/thaw. Keep on ice while in use. Use within two months.
- **ECE-1 substrate:** Store at -20 ºC, avoid light. Bring to room temperature before use.
- **ECE-1 Inhibitor Mix:** Reconstitute in 100 µl dH_2O and mix thoroughly. Store at -20°C. Keep on ice while in use. Use within two months.
- **MCA Standard:** Light sensitive. Store at -20°C. Bring to room temperature before use.

VII. Endothelin Converting Enzyme 1 Activity Assay Protocol:

1. ECE-1 Inhibitor, Sample Preparation:

a. ECE-1 Inhibitor:

i. Prepare a 100-fold dilution of ECE-1 Inhibitor Mix (i.e. Dilute 2 µl of ECE-1 Inhibitor Mix with 198 µl ECE-1 Assay Buffer).

ii. Further prepare a 10-fold dilution of ECE-1 Inhibitor Mix (i.e. Dilute 10 µl of prepared ECE-1 Inhibitor Mix (see **Step 1.a.i**) with 90 µl ECE-1 Assay Buffer).

iii. Add 10 µl of Diluted ECE-1 Inhibitor Mix (see **Step 1.a.ii**) into desired well(s) in a 96-well white plate labeled as **Sample Background Control;** add 10 µl of ECE-1 Assay Buffer into the parallel well(s) as **Sample.**

Note: Do not store the Diluted Inhibitor Mix. Prepare fresh dilutions prior to the experiments.

b. Sample Preparation:

Homogenize cells (~1X10^6) or tissue (~50 mg) with 200 µl of iced-cold ECE-1 Assay Buffer containing protease inhibitor cocktail (Cat. # K271 or equivalent) and keep on ice for 10 min. Centrifuge samples at 12,000 *x g* at 4 ºC for 10 min. and collect the supernatant. Dilute samples 5-20 fold with ECE-1 Assay Buffer. Add 2-10 µl of diluted sample into wells assigned as **Sample**

Background Control and **Sample**. For positive control, add 4-10 µl of ECE-1 into desired well(s). Adjust the volume of Positive Control, Sample, and Sample Background Control wells to **80 µl/well** with ECE-1 Assay Buffer and **incubate the plate at 37 °C for 20 minutes**, protected from light.

Note: For unknown samples, we recommend doing pilot experiment and testing several doses to ensure the readings are within the Standard Curve range and the signal kinetics are within the linear range.

2. **Standard Curve Preparation:** Prepare a 25 µM MCA Standard by adding 2 µl of 5 mM MCA Standard to 398 µl ECE-1 Assay Buffer. Add 0, 2, 4, 6, 8, 10 µl of 25 µM MCA standard into a series of wells of white 96-well plate to generate 0, 50, 100, 150, 200, 250 pmol of MCA/well respectively. Adjust the volume to 100 µl/well with ECE-1 Assay Buffer.

   Note: Equilibrate the Standard Solution to 37 ºC before adding to the wells.

3. **ECE-1 Substrate Mix Preparation:** Prepare a 50-fold dilution of ECE-1 Substrate Stock Solution (i.e. Dilute 2 µl of ECE-1 Substrate with 98 µl of ECE-1 Assay Buffer), vortex briefly. **After sample(s) were incubated at 37 ºC for 20 min (see step 1.b)**, add 20 µl of Diluted ECE-1 Substrate Mix to each well containing test sample(s) labeled as Sample, Sample Background Control and ECE-1 positive control(s).

   Note:

   a. Equilibrate the Substrate Mix to 37 ºC before adding to the wells.

   b. *Do not add Substrate Mix to the standards*.

4. **Measurement:** Measure fluorescence (Ex/Em: 320/420 nm) in kinetic mode at 37 ºC for 30-40 min. 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). The MCA standards can be read in end-point mode (i.e. at the end of the experiment) (see step 2) at Ex/Em= 320/420 nm at 37 °C.

   Note: Incubation time depends on the ECE-1 activity in samples. Longer incubation time may be required for samples having low ECE-1 activity.

5. **Calculation:** Subtract 0 Standard reading from all standard readings. Plot the MCA Standard Curve. For each reaction well (Sample and Sample Background Control), 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). Apply each well ΔRFU to the MCA Standard Curve to get amounts of MCA generated during the reaction time (t_2-t_1). Calculate the background corrected sample MCA formed (B, in pmol) by subtracting the amount of MCA formed by Sample Background Control from the amount of MCA formed by Sample and calculate the activity of ECE-1 activity in the sample as:

$$\mathbf{B}\ (\text{pmol}) = \mathbf{B}_{\text{Sample}} - \mathbf{B}_{\text{Sample Background Control}}$$

$$\textbf{Sample ECE-1 Specific Activity} = \mathbf{B}/(\Delta t * V * P) \times D = \text{pmol/min/mg} = \mu\text{U/mg}$$

Where: **B** = MCA from Standard Curve (pmol)
Δt = Reaction time (min.)
V = Sample volume added into the reaction well (µl)
P = Sample Concentration in µg-protein/µl
D = Dilution Factor

Unit Definition: One unit of ECE-1 activity is the amount of enzyme that generates 1.0 µmol of MCA per min., under the assay conditions at 37 ºC.

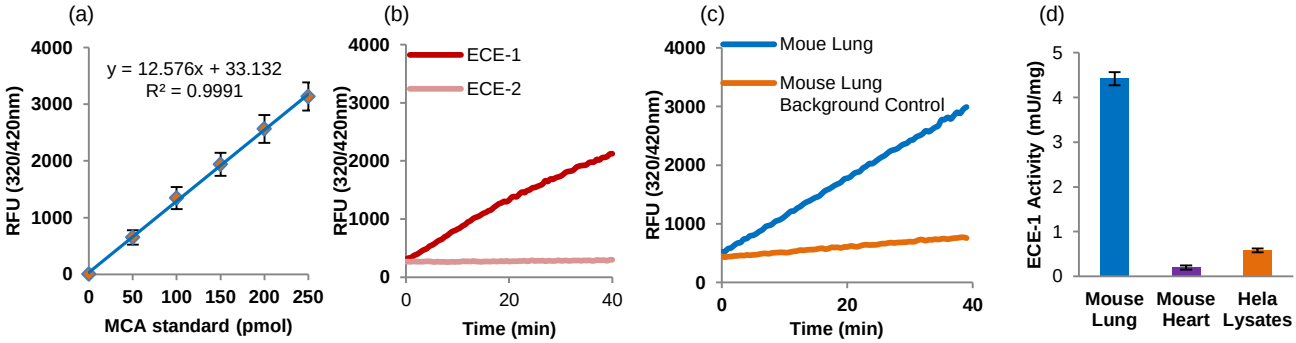

Figure: (a) MCA Standard Curve. **(b)** Measurement of purified ECE-1 (5.5 ng) and ECE-2 (12 ng) activities. The kit can effectively discriminate ECE-1 activity from ECE-2 activity. **(c)** ECE-1 activity in Mouse Lung (1.1 µg protein) with or without Inhibitor Mix. **(d)** Measurement of ECE-1 Activity in Mouse Lung Extracts (1.1 µg protein), Mouse Heart Extracts (1.5 µg protein) and HeLa lysates (1.5 µg protein). All assays were performed following kit protocol.

VIII. RELATED PRODUCTS:

Human CellExp™ ECE1, Human Recombinant (P1227)
Neprilysin Activity Assay Kit (K487-100)
Neprilysin Inhibitor Screening Kit (K996)
Angiotensin I Converting Enzyme (ACE1) Activity Assay Kit (K227)
Angiotensin I Converting Enzyme Inhibitor Screening Kit (K228)
Angiotensin II Converting Enzyme Inhibitor Screening Kit (K310)
Angiotensin II Converting Enzyme (ACE2) Activity Assay Kit (K897)

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