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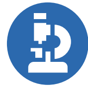
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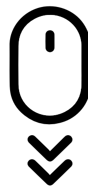

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ENPP1/ENPP3 Cell-Based Activity Assay Kit

Item No. 702080

www.caymanchem.com
Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400544	ENPP1/ENPP3 Cell-Based Assay Buffer (10X)	1 vial/5 ml	4°C
400545	ENPP1/ENPP3 Dual Inhibitor Mix	1 vial/100 µl	-20°C
400546	ENPP1 Inhibitor C	1 vial/100 µl	-20°C
400547	ENPP1/ENPP3 Substrate (TG-mAMP)	1 vial/60 µl	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence with excitation and emission wavelengths of 485 and 520 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. Microcentrifuge tubes
4. Centrifuge (suspension cells only)
5. DMSO
6. A 96-well, black with clear bottom, tissue culture plate
7. A source of pure water; glass-distilled water or HPLC-grade water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*

INTRODUCTION

Background

Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) and ENPP3 are type II transmembrane glycoproteins with nucleotide pyrophosphatase and phosphodiesterase enzymatic activities.[1,2] ENPP1 and ENPP3 are expressed in a wide variety of tissues with ENPP1 also expressed in pancreatic islets, chondrocytes, and lymphocytes, among other cell types, and ENPP3 on the surface of basophils and mast cells.[3-5] ENPP1 is a critical regulator of purinergic signaling that catalyzes the hydrolysis of ATP to AMP, generates inorganic pyrophosphates, and catalyzes 2'3'-cGAMP hydrolysis.[1,6] ENPP3 is selective for ATP over other nucleotide triphosphates but also hydrolyzes nucleotide sugars and diadenosine polyphosphates.[2]

ENPP1 has roles in hypoxia, ischemia, and apoptosis, as well as cell proliferation, migration, and differentiation, and its hydrolysis of 2'3'-cGAMP facilitates immunosuppression in the tumor microenvironment.[1,6] Loss-of-function mutations in *ENPP1* are associated with various calcification-related disorders.[1,7] ENPP3 is involved in cell migration, cell differentiation, and mediating the allergic response and has been used as a biomarker of allergen sensitivity.[8] ENPP3 is highly expressed in the kidney of patients with renal cell carcinoma (RCC), and an antibody-drug conjugate inhibitor of ENPP3 inhibits tumor growth in RCC mouse xenograft models.[5] ENPP1 and ENPP3 are potential therapeutic targets in the treatment of cancer and immunological diseases.

About This Assay

Cayman's ENPP1/ENPP3 Cell-Based Activity Assay Kit provides a robust and easy-to-use platform for determining the activities of ENPP1 and/or ENPP3 in live cells. The assay uses an ENPP1/ENPP3-specific fluorogenic substrate, TG-mAMP.[9] ENPP1/ENPP3 cleaves this substrate generating free Tokyo Green™, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 485 and 520 nm, respectively. Respective ENPP1 and ENPP3 activities can be distinguished from one another by the use of specific ENPP1 inhibitor, ENPP1 Inhibitor C or the ENPP1/ENPP3 Dual Inhibitor Mix provided in this kit.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **ENPP1/ENPP3 Cell-Based Assay Buffer (1X) – (Item No. 400544)**

   This vial contains 5 ml of concentrated ENPP1/ENPP3 Cell-Based Assay Buffer. Add 5 ml of ENPP1/ENPP3 Cell-Based Assay Buffer (10X) to 45 ml of pure water. The solution, diluted or undiluted, will be stable for at least two weeks when stored at 4°C. Freeze at -20°C for long-term storage.

2. **ENPP1/ENPP3 Dual Inhibitor Mix – (Item No. 400545)**

   This vial contains 100 µl of a mixture of ENPP1 and ENPP3 inhibitors in DMSO. The ENPP1/ENPP3 Dual Inhibitor Mix is ready to use as supplied. If all of the ENPP1/ENPP3 Dual Inhibitor Mix will not be used at one time, the inhibitor mix can be stored at -20°C, limiting freeze-thaw cycles.

3. **ENPP1 Inhibitor C – (Item No. 400546)**

   This vial contains 100 µl of 4,500 µM of ENPP1 Inhibitor C in DMSO, which is ready to use as supplied. If all of the ENPP1 Inhibitor will not be used at one time, the inhibitor can be stored at -20°C, limiting freeze-thaw cycles.

4. **ENPP1/ENPP3 Substrate (TG-mAMP) – (Item No. 400547)**

   Each vial contains 60 µl of an ENPP1/ENPP3 Substrate (TG-mAMP) solution. Mix 50 µl ENPP1/ENPP3 Substrate with 2,450 µl of ENPP1/ENPP3 Cell-Based Assay Buffer (1X) to make a substrate working solution. This is a sufficient volume of substrate working solution to assay at least 100 wells. If all of the substrate will not be used at one time, the undiluted substrate can be stored at -20°C protected from light, limiting freeze-thaw to 3 cycles. The undiluted substrate will be stable when stored at -20°C for up to 6 months.

Sample Preparation

NOTE: Fetal bovine serum (FBS) contains ENPPs that may generate a high background reading when used in the culture medium. Ensure that cells are thoroughly rinsed during the wash steps of the assay procedure.

Cell Culture Preparation

Culture cells in a CO_2 incubator at 37°C for 24 hours or according to the user's typical experimental protocol. It is recommended that each experimental sample be performed at least in duplicate (triplicate is preferred). Follow steps 1 and 2 on pages 10 and 11 for adherent cells and suspension cells, respectively.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is recommended that each sample be assayed in triplicate and that the contents of each well are recorded on the template sheet provided on page 18. A typical layout of samples to be measured in triplicate is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	I	I	I	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW = Background Wells
I = Inhibited Wells
1-30 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all of the wells.
- Use the diluted assay buffer in the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- Up to 30 samples can be assayed in triplicate or up to 46 in duplicate.
- The assay is performed at 37°C.
- Monitor the fluorescence at excitation and emission wavelengths of 485 and 520 nm, respectively.

Performing the Assay

1. **Adherent Cells**

   Seed 2,000 to 15,000 cells per well into a black 96-well tissue culture-treated plate with a clear bottom. Be sure to seed approximately the same number of cells in each well. Allow adequate time for the cells to adhere. Wash the cells twice with at least 100 µl of ENPP1/ENPP3 Cell-Based Assay Buffer (1X). Carefully aspirate the remaining liquid without detaching the cells. Add 79 µl of ENPP1/ENPP3 Cell-Based Assay Buffer (1X) to each well.

2. **Suspension Cells**

   Wash 2,000 to 15,000 cells per well in a 96-well V-bottom plate and centrifuge at 500 x g. Be sure to use approximately the same number of cells in each well. Wash the cells twice with at least 100 µl of ENPP1/ENPP3 Cell-Based Assay Buffer (1X). Carefully aspirate the remaining liquid without disturbing the cell pellet. Transfer cells into a black 96-well tissue culture-treated plate with a clear bottom in a total volume of 79 µl ENPP1/ENPP3 Cell-Based Assay Buffer (1X) per well.

3. **Sample Wells**

   Add 1 µl of DMSO to the designated wells on the plate. Mix all wells thoroughly.

4. **Inhibited Sample Wells**

   Add 1 µl of ENPP1 Inhibitor C or ENPP1/3 Dual Inhibitor Mix to the designated wells on the plate. Mix all wells thoroughly.

5. **Background Wells**

   Add 80 µl of ENPP1/ENPP3 Cell-Based Assay Buffer (1X) to at least two empty wells.

6. Cover the plate and incubate at 37°C for 5 minutes.
7. Remove the plate cover and quickly initiate the reactions by adding 20 µl of substrate working solution to all of the wells being used.
8. Immediately measure the fluorescence using excitation and emission wavelengths of 485 and 520 nm, respectively, every 5 minutes for 60 minutes at 37°C. If ENPP1/ENPP3 activity is low, continue reading for up to 120 minutes.

Well	Substrate Working Solution	Assay Buffer (1X)	Inhibitor C	Inhibitor Mix	DMSO
Uninhibited Sample	20 µl	79 µl	--	--	1 µl
Sample with ENPP1 Inhibitor C	20 µl	79 µl	1 µl	--	--
Sample with ENPP1/ENPP3 Dual Inhibitor Mix	20 µl	79 µl	--	1 µl	--
Background Well	20 µl	80 µl	--	--	--

Table 1. Pipetting summary

Calculations

ENPP1 and/or ENPP3 activities can be qualitatively determined by observing the change in fluorescence over time (see Figures 2 and 3 on pages 13-14). It is recommended to normalize the endpoint fluorescence intensity against cell density to account for the variation in cell count per well when comparing ENPP1 and/or ENPP3 activities between samples and treatment conditions. See Figure 3 on page 14 for an example.

ANALYSIS

Performance Characteristics

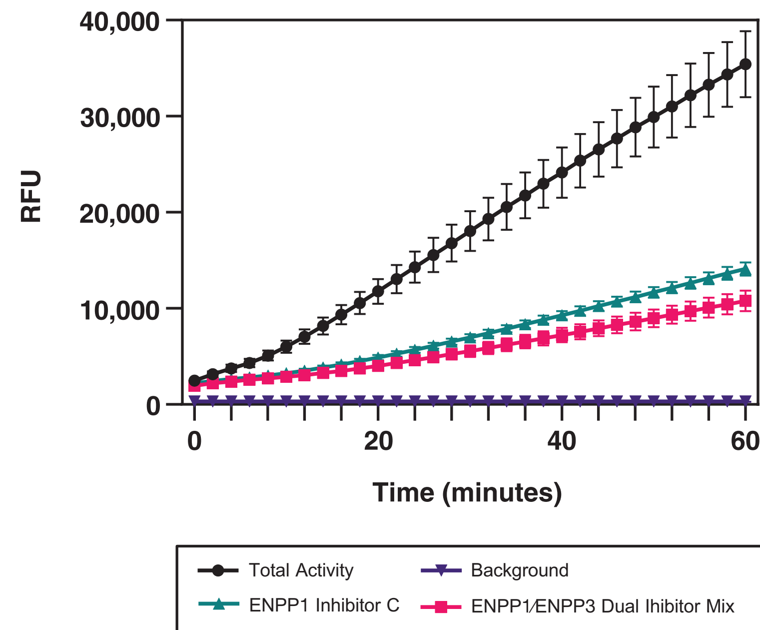

Figure 2. Activity of ENPP1/ENPP3 in HepG2 cells with and without inhibitors

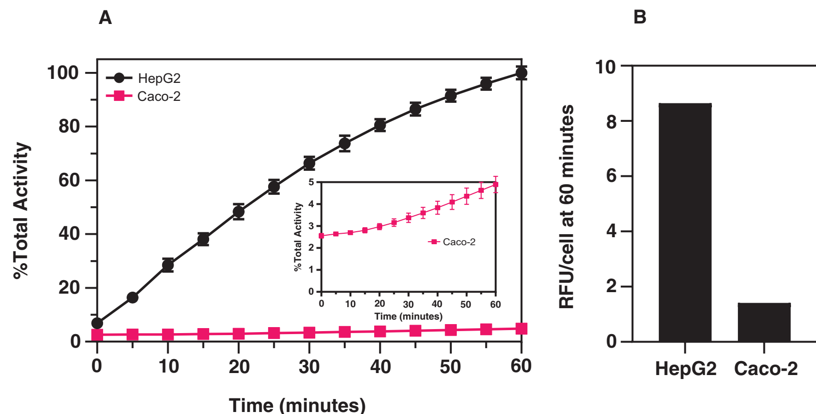

Figure 3. Activity of ENPP1/ENPP3 in HepG2 and Caco-2 cells . (A) Normalized ENPP1/ENPP3 activity in HepG2 *vs.* Caco-2 cells. **(B)** The endpoint fluorescence values of HepG2 and Caco-2 cells at 60 minutes were normalized against their respective cell densities. ENPP1 and ENPP3 expression levels are higher in HepG2 cells than in Caco-2 cells. See the Human Protein Atlas website for more information on the expression levels of these proteins.

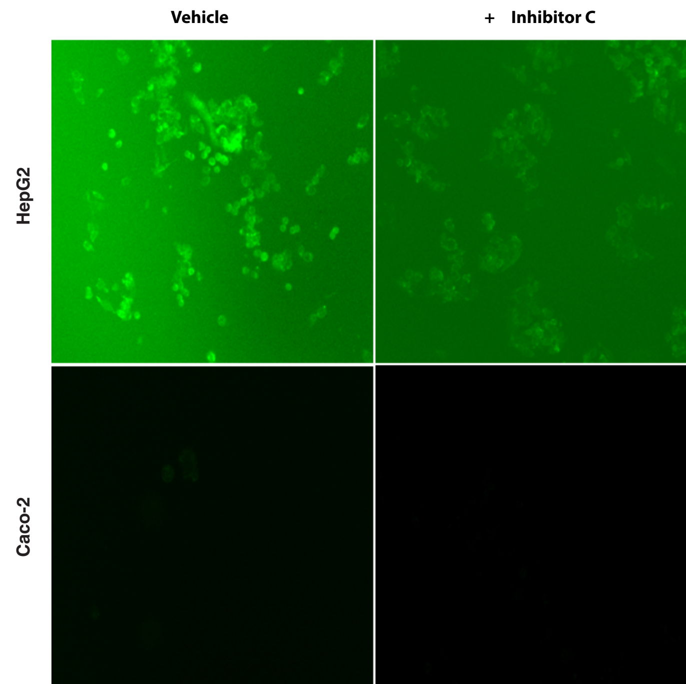

Figure 4. Representative images showing ENPP1 and/or ENPP3 activities in HepG2 and Caco-2 cells and their inhibition by ENPP1 Inhibitor C. HepG2 and Caco-2 cells were treated with 45 µM of ENPP1 Inhibitor C for 5 minutes at 37°C, followed by a one-hour incubation with the ENPP1/ENPP3 substrate TG-mAMP.

Interferences

FBS interferes with the assay. It may generate a high background reading when used in the culture medium due to its high ENPP content.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No difference in fluorescence between inhibited and uninhibited wells	A. Inhibitor was not added to the wells B. ENPP1/ENPP3 activity is too low to detect	Make sure to add all the components to the wells and re-assay
High fluorescence in inhibited wells	Cells used have higher ENPP1/ENPP3 expression than what the inhibitor can block	Use a higher concentration of inhibitor and run the assay again or seed cells at a lower density

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	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES

Warranty and Limitation of Remedy

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