ENPP1 Fluorescent Inhibitor Screening Assay Kit

Cat. No. KITZ-003 **Lot. No.** (See product label)

SPECIFICATION

Product Overview	ENPP1 Fluorescent Inhibitor Screening Assay Kit provides a stability and convenience platform for identifying novel inhibitors of human ENPP1, an ectonucleotide pyrophosphatase and phosphodiesterase implicated in multiple biological pathways, including the cGAS/STING pathway, and bone mineralization. Tokyo Green™-mAMP (TG-mAMP), an ENPP1-specific fluorogenic substrate, is included in the kit. ENPP1 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. Besides, the potent and reversible ENPP1 inhibitor ENPP1 Inhibitor C Assay Reagent is included as a positive control.
Applications	Screen for inhibitors of human ENPP1
Storage	-80°C
Shipping	Dry ice
Kit Components	ENPP1 Assay Buffer (10X): 1 vial/5ml, -20°C; ENPP1 Enzyme (human, recombinant): 1 vial/40ul, -80°C; ENPP1 Substrate (TG-mAMP): 1 vial/250ul, -20°C; ENPP1 Inhibitor C Assay Reagent: 1 vial/50ul, -20°C; Half-Volume 96-Well Solid Plate (black): 1 plate, RT; 96-Well Cover Sheet: 1ea, RT;

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Materials Required but Not Supplied	1.A plate reader with the ability to measure fluorescence with excitation and emission wavelengths of 485 and 520 nm, respectively. 2.Adjustable pipettes; multichannel or repeating pipettor recommended. 3.A source of ultrapure water, with a resistivity of 18.2 MΩ•cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water-glass-distilled or deionized- may not be acceptable. 4.Microcentrifuge tubes
Detection method	Fluorescent reader (Ex/Em = 485/520 nm)
Assay Protocol	Sample preparation: All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted ENPP1Assay Buffer at a concentration 10X the desired final assay concentration (e.g., for 30uM final assay concentration, a 300uM stock should be made). This solution may contain up to 20% DMSO or short-chain alcohols (e.g., MeOH, EtOH). Dimethyl formamide (DMF) is not recommended as a solvent. The final concentration of organic solvents in the assay will then be ≤2%. Reagent preparation: 1.ENPP1 Assay Buffer (1X): Mix 2 ml of ENPP1 Assay Buffer (10X) with 18 ml of ultrapure water to make 20 ml of ENPP1 Assay Buffer (1X). The ENPP1 Assay Buffer(1X) should be discarded if not used within the same day. Once thawed, the ENPP1 Assay Buffer (10X) may be stored at 4°C. 2.ENPP1 Substrate (TG-mAMP): Mix 200ul ENPP1 Substrate (TG-mAMP) with 1.8 ml ENPP1 Assay Buffer (1X). The diluted substrate will be stable at room temperature (RT) for four hours. If all of the ENPP1 Substrate (TG-mAMP) will not be used at one time, aliquot the undiluted substrate and store at -20°C. 3.ENPP1 Enzyme (human, recombinant): ENPP1 Enzyme (human, recombinant) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 25ul of ENPP1Enzyme (human, recombinant) with 1.975 ml ENPP1 Assay Buffer (1X). It is recommended that the enzyme be diluted immediately prior to performing the assay.

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The diluted enzyme loses 30% of its activity when stored on ice for one hour. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

4.ENPP1 Inhibitor C Assay Reagent: This vial contains 50ul of 6 mM ENPP1 Inhibitor C Assay Reagent in DMSO, which can be used as a positive control. Mix 5ul of ENPP1 Inhibitor C Assay Reagent with 95ul ENPP1 Assay Buffer (1X) to make a 300uM working solution. If all of the ENPP1 Inhibitor C Assay Reagent will not be used at one time, aliquot the undiluted inhibitor and store at -20°C, limiting freeze-thaw cycles.

Plate set up:

The 96-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control ENPP1 Inhibitor C Assay Reagent, be assayed in triplicate. It is suggested that the contents of each well be recorded on a template sheet. A typical layout of samples to be measured in triplicate is shown in Figure below:

BW-Background wells; A-100% Initial activity wells; PC-Positive control wells; 1-29-Inhibition wells.

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 100ul in all the wells.
Use the diluted assay buffer in the assay.
All reagents should be prepared as described above. The ENPP1 enzyme should be kept on ice and all other reagents should be kept at RT before beginning 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 at the user's discretion to do so.
The assay is performed at RT.
Monitor the fluorescence with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.
Performing the Assay:
1.Background Wells: add 70ul ENPP1 Assay Buffer(1X) and 10ul of solvent (the same solvent concentration used to dissolve the unknown inhibitor and the positive controlENPP1 Inhibitor C Assay Reagent) to three wells. If different solvents are to be assayed at the same time, separate sets of background wells should be run for each solvent.
2.100% Initial Activity Wells: add 50ul of ENPP1 Assay Buffer (1X), 20ul of ENPP1 Enzyme, and 10ul of solvent to three wells. If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent.
3.Inhibitor/Positive Control Wells: add 50ul ENPP1 Assay Buffer (1X), 20ul of ENPP1 Enzyme, and 10ul of unknown inhibitor or the 300uM positive control, ENPP1 Inhibitor C Assay Reagent working solution, to three wells.
NOTE: To determine an IC50 value for an inhibitor, multiple concentrations of the inhibitor should be tested in the assay.
4.Incubate for 5 minutes at RT.

5.Initiate the reactions by adding 20ul of ENPP1 Substrate (TG-mAMP) to all the wells being used. Mixing the contents is not necessary.
6.Cover the plate with the 96-Well Cover Sheet and incubate for twenty minutes at RT protected from light.
7.Remove the plate cover and read the plate with an excitation wave length of 485 nm and an emission wavelength of 520 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples*.
*If desired, the assay may be read kinetically rather than as an endpoint. Reading the assay kinetically may increase signal-to-background. The fluorescence should be measured at least once per minute at RT for 30 minutes. Determine the initial rate based on the linear portion of the kinetic curve. Calculations can be performed as shown in the calculation section substituting initial rates for average fluorescence.

Calculations:
1.Determine the average fluorescence (AF) of each sample.
2.Subtract the AF of the background wells from the AF of the 100% initial activity and inhibitor wells. These are the corrected values.
3.Determine the percent inhibition or percent activity for each inhibitor using one of

the following equations:

4.Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the lC50 value (the concentration at which there is 50% inhibition) of the inhibitor.

Performance Characteristics:

Z' Factor:

Z' factor is a term used to describe the robustness of an assay, which is calculated using the equation below.

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z’ factor for ENPP1 Fluorescent Inhibitor Screening Assay Kit was determined to be 0.82 for a 96-well plate.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO or short-chain alcohols (e.g., MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤ 2%. A titration of organic solvents showed that the signal changes with

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increasing solvent concentration, so the proper vehicle control should be included in the assay.

Precision:

Intra-assay precision was determined by analyzing 3, 7, and 7 measurements of the background, vehicle, and 30uM ENPP1 Inhibitor C Assay Reagent on the same day. The intra-assay coefficients of variation were 2,8, and 9%, respectively. The intra-assay coefficient of variation for the IC50 value of eleven inhibition curves performed on the same day was 4%.

Inter-assay precision was determined by analyzing inhibition with ENPP1 Inhibitor C Assay Reagent in six separate assays on different days. The inter-assay coefficient of variance for the IC50 value was 11%.

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