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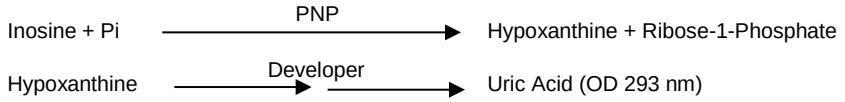
Purine Nucleoside Phosphorylase Activity Assay Kit (Colorimetric)

(Catalog # K768-100; 100 assays; Store at -20°C)

I. Introduction:

Purine Nucleoside Phosphorylase (PNP) (E.C. 2.4.2.1.) is an enzyme involved in purine metabolism and it catalyzes the cleavage of the glycosidic bond of ribo- or deoxyribonucleosides, in the presence of inorganic phosphate as a second substrate, to generate the purine base and ribose-1-phosphate or deoxyribose-1-phosphate. It is one of the enzymes of the nucleotide salvage pathways that allow the cell to produce nucleotide monophosphates when the de novo synthesis pathway has been interrupted or is non-existent (as is the case in the brain). PNP is a cytosolic enzyme. PNP deficiency disease leads to toxic buildup of deoxyguanosine in T-cells leading to T-lymphocytopenia with no apparent effects on B-lymphocyte function. Inhibition of PNP is an important target for chemotherapeutic applications and treatment of T-cell mediated autoimmune diseases. PNP deficiency is also associated with neurological problems. In BioVision's Purine Nucleoside Phosphorylase Activity Assay, hypoxanthine formed by the breakdown of inosine is further converted to uric acid using a developer. The uric acid is measured at a wavelength of 293 nm. Limit of quantification is 0.1 µU recombinant Purine Nucleoside Phosphorylase.



II. Application:

- Detection of Purine Nucleoside Phosphorylase activity in variety of samples

III. Sample Type:

- Purified recombinant protein
- Cell and tissue lysate

IV. Kit Contents:

Components	K768-100	Cap Code	Part Number
PNP Assay Buffer (10x)	10 ml	WM	K768-100-1
Developer	1 Vial	Blue	K768-100-2
Inosine Substrate	200 µl	Brown	K768-100-3
Hypoxanthine Standard (10 mM)	100 µl	Yellow	K768-100-4
PNP Positive Control	1 Vial	Green	K768-100-5
U.V. transparent plate (96-well)	1	–	K768-100-6

V. User Supplied Reagents and Equipment:

- Microplate reader capable of absorbance measurement
- Protease Inhibitor Cocktail (Cat. # K271 or its equivalent)
- Dounce Homogenizer

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.

- **PNP Assay Buffer (10x):** Make 1x buffer by adding one part 10x Assay Buffer to nine parts deionized water. Store at -20°C or 4°C. Bring to room temperature before use.
- **Developer:** Reconstitute with 210 µl 1x PNP Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents in the bottom of the tube. Aliquot and store at -20°C. Avoid repeated freeze/thaw.
- **Inosine Substrate:** Aliquot and store at -20°C. Avoid repeated freeze/thaw.
- **PNP Positive Control:** Reconstitute with 22 µl 1x PNP Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents in the bottom of the tube. Aliquot and store at -20°C. Avoid repeated freeze/thaw

VII. Purine Nucleoside Phosphorylase Assay Protocol:

1. **Sample Preparation:** Rinse tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80°C) to a pre-chilled homogenizer. Add 300 µl cold 1x PNP Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice. Transfer the tissue homogenate to a cold microfuge tube.

   To prepare cell extract, add 150-300 µl cold 1x PNP Assay Buffer containing protease inhibitor cocktail (not provided) to 1-5 x 10^6 fresh or frozen cells and pipette several times to disrupt the cells. Transfer cell homogenate including cell debris to a cold microfuge tube and agitate on a rotary shaker at 4°C for at least 15 min.

   Centrifuge the tissue or cell homogenate at 10,000 X g, 4°C for 15 min. Transfer the clarified supernatant to a fresh pre-chilled tube & store on ice. Use lysates immediately to assay PNP activity.

   Note: Lysates can be aliquoted and snap frozen in liquid nitrogen before storing at -80°C. Avoid freeze/thaw.

2. **Hypoxanthine Standard:** Dilute Hypoxanthine Standard to 1 mM by adding 10 µl of 10 mM Hypoxanthine Standard to 90 µl 1x PNP Assay Buffer. Add 0, 2, 4, 6, 8, and 10 µl of diluted 1 mM Hypoxanthine Standard into a series of wells in the provided 96-well plate to generate 0, 2, 4, 6, 8, and 10 nmol/well Hypoxanthine Standard. Adjust the volume to 50 µl/well with 1x PNP Assay Buffer.

3. **Purine Nucleoside Phosphorylase Activity Assay:** Add 2-50 µl of sample into desired well(s) in the provided 96-well plate. For Positive Control, add 2 µl Positive Control for the assay. Make up the volume of samples and Positive Control to 50 µl/well with 1x PNP Assay Buffer. Add 50 µl 1x PNP Assay Buffer to one well as reagent Background Control.

   Notes:

   a. For unknown samples, we suggest doing a pilot experiment and testing several doses to ensure the readings are within the Standard Curve range.

   b. Small molecules such as xanthine and hypoxanthine in the samples will contribute to the background. If the background level is too high, remove these molecules by passing through a desalting column (# 6564) or by buffer exchange using a 10kDa spin column (Cat # 1997). Use this treated sample for the assay. **Optional:** Prepare a parallel sample well as sample background control to ensure that the small molecules are removed by either using a desalting or spin column.

4. **Reaction Mix:** Prepare enough reagents for the number of assays to be performed. Make 50 µl of Reaction Mix and Background Control Mix containing:

	Reaction Mix	Background Control Mix
1x PNP Assay Buffer	46 µl	48 µl
Developer	2 µl	2 µl
Inosine Substrate	2 µl	-----

   Add 50 µl of Reaction Mix into each sample, reagent background control and Positive Control wells and 50 µl of Background Control mix to Standards and sample background control well(s). Mix well.

5. **Measurement:** Measure absorbance (OD 293 nm) in kinetic mode for at least 30 min. at room temperature. Choose two time points (T_1 & T_2) in linear range (can be as short as 2 min.) of plot and obtain corresponding absorbance for sample (A_{S1} and A_{S2}) and reagent background control (A_{BG1} and A_{BG2}). Read the Hypoxanthine Standard Curve along with the samples.

6. **Calculations:** Subtract 0 Standard reading from all Standard Readings. Plot the Hypoxanthine Standard Curve. Subtract reagent background control reading from sample reading. Apply the ΔOD $[(A_{S2-} A_{BG2}) - (A_{S1-} A_{BG1})]$ to the Standard Curve to get B nmol of Hypoxanthine generated by the sample during the reaction time ($\Delta T = T_2 - T_1$).

$$\textbf{Sample's PNP Activity} = \frac{\mathbf{B}}{\Delta T \times \mu g\ of\ protein}\ \textbf{x DF = nmol/min/µg = mU/µg}$$

Where: **B** is Hypoxanthine amount from Standard Curve (nmol).
ΔT is the reaction time (min.)
µg of protein is the amount of protein/well in µg
DF is the dilution factor of the sample

Sample PNP Activity can also be expressed as U/mg (µmoles/min hypoxanthine generated per mg) of protein.

Unit Definition: One unit of Purine Nucleoside Phosphorylase Activity is the amount of enzyme that hydrolyzes inosine to yield 1.0 µmol of hypoxanthine/min. at room temperature.

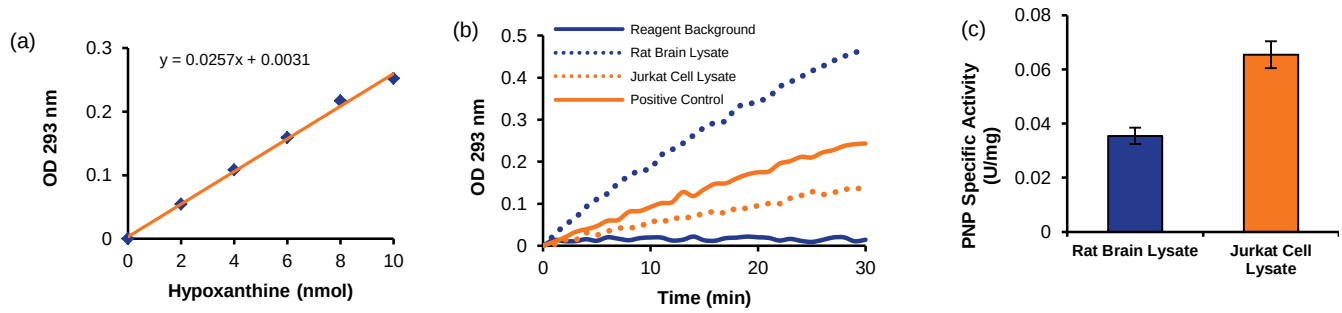


Figure: (a) Hypoxanthine Standard Curve, (b) Purine Nucleoside Phosphorylase Activity in Jurkat cell (T-lymphocyte) lysate (3 µg), rat brain lysate (19 µg), and Positive Control (c) PNP specific activity in Jurkat cell lysate and rat brain lysate. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Purine Nucleoside Phosphorylase Activity Assay Kit (Fluorometric) (K767)
Xanthine Oxidase Colorimetric/Fluorometric Assay Kit (K710)
Xanthine/Hypoxanthine Colorimetric/Fluorometric Assay Kit (K685)
Adenosine Activity Assay (Fluorometric) (K327)
Ammonia Colorimetric Assay Kit (K370)
Adenosine Deaminase Activity Assay (Fluorometric) (K768)
Inosine Fluorometric Assay Kit (K712)
Uric Acid Colorimetric/Fluorometric Assay Kit (K608)
Ammonia Colorimetric Assay Kit II (K470)

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