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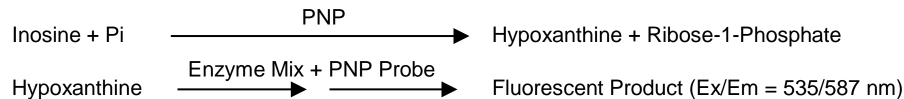
Purine Nucleoside Phosphorylase Activity Assay Kit (Fluorometric)

4/15

(Catalog # K767-100; 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 allows 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 from the breakdown of inosine is detected via a multi-step reaction, resulting in the generation of an intermediate that reacts with the PNP Probe. The fluorescent product is measured at Ex/Em = 535/587 nm. Limit of quantification is 0.005 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	K767-100	Cap Code	Part Number
PNP Assay Buffer (10x)	10 ml	WM	K767-100-1
Enzyme Mix	1 Vial	Blue	K767-100-2
Inosine Substrate	200 µl	Brown	K767-100-3
PNP Probe (in dry DMSO)	200 µl	Red	K767-100-4
Hypoxanthine Standard (10 mM)	100 µl	Yellow	K767-100-5
PNP Positive Control	1 Vial	Green	K767-100-6

V. User Supplied Reagents and Equipment:

- 96-well plate with flat bottom. White plate is preferred for this assay.
- Fluorescence microplate reader
- 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 37°C before use.
- **Enzyme Mix:** Reconstitute with 210 µl 1x PNP Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents at 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 200 µl 1x PNP Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents at 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 -20°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. Further dilute the Hypoxanthine Standard to 10 µM by adding 10 µl of 1 mM Hypoxanthine to 990 µl 1x PNP Assay Buffer. Add 0,

2, 4, 6, and 8 µl of diluted 10 µM Hypoxanthine Standard into a series of wells in 96-well plate to generate 0, 20, 40, 60, and 80 pmol/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 96-well plate. For Positive Control, dilute Positive Control 5x in 1x PNP Assay Buffer and add 2-4 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 high, remove these molecules by passing through a desalting column (Cat. # 6564) or by buffer exchange using a 10kDa spin column (Cat. # 1997). Use this modified 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	45 µl	47 µl
Enzyme Mix	2 µl	2 µl
PNP Probe	1µl	1 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 fluorescence (Ex/Em = 535/587 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 RFU for sample (RFU_{S1} and RFU_{S2}) and reagent background control (RFU_{BG1} and RFU_{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 ΔRFU [(RFU_{S2}- RFU_{BG2}) – (RFU_{S1}- RFU_{BG1})] to the Standard Curve to get B pmol of Hypoxanthine generated by the sample during the reaction time ($\Delta T = T_2 - T_1$).

   Note: Sample background control reading should be less than reagent background control reading. We recommend removing the small molecules again using desalting column (Cat. # 6564) or a 10 kDa spin column (Cat. # 1997) if sample background control reading is higher than reagent background control

$$\text{Sample's PNP Activity} = \frac{B}{\Delta T \times \mu g\ of\ protein} \times DF = \text{pmol/min/µg} = \text{µU/µg} = \text{mU/mg}$$

Where: **B** is Hypoxanthine amount from Standard Curve (pmol).
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 mU/mg (nmoles/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.

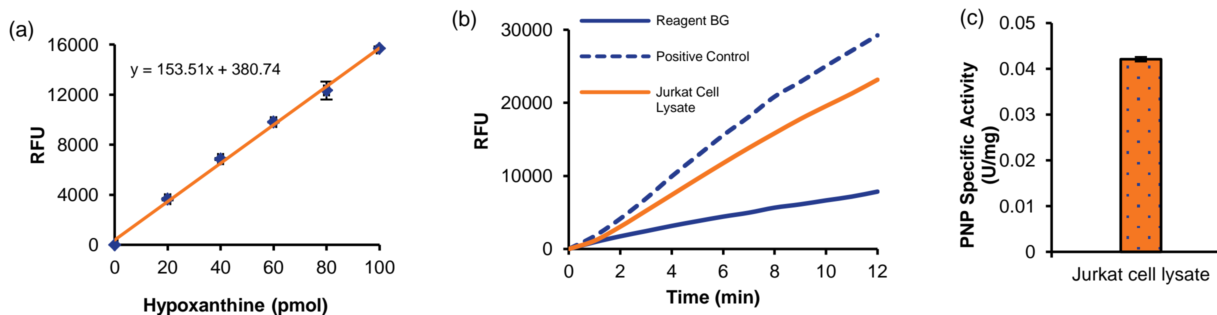


Figure: (a) Hypoxanthine Standard Curve, (b) Purine Nucleoside Phosphorylase Activity in Jurkat Cell (T-lymphocyte) lysate (315 ng) and Positive Control; BG: Background (c) PNP specific activity in Jurkat Cell lysate. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Xanthine Oxidase Colorimetric/Fluorometric Assay Kit (K710)
Xanthine/Hypoxanthine Colorimetric/Fluorometric Assay Kit (K685)
Adenosine Deaminase Activity Assay Kit (Fluorometric) (K328)
Ammonia Colorimetric Assay Kit (K370)
Inosine Fluorometric Assay Kit (K712)
Uric Acid Colorimetric/Fluorometric Assay Kit (K608)
Ammonia Colorimetric Assay Kit II (K470)

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