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Product Information

Purine Nucleoside Phosphorylase Activity Assay Kit (Fluorometric)

Catalog Number **MAK288**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Purine Nucleoside Phosphorylase (PNP, E.C. 2.4.2.1.) is an enzyme involved in purine metabolism. PNP 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).

Purine Nucleoside Phosphorylase is a cytosolic enzyme. PNP deficiency disease leads to toxic buildup of deoxyguanosine in T-cells leading to T-lympho-cytopenia 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 this 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 λ_{ex} = 535 nm/λ_{em} = 587 nm. Limit of quantification is 0.005 μU of recombinant Purine Nucleoside Phosphorylase.

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

Components

The kit is sufficient for 100 assays in 96 well plates.

PNP Assay Buffer (10×) Catalog Number MAK288A	10 mL
Enzyme Mix Catalog Number MAK288B	1 vial
Inosine Substrate Catalog Number MAK288C	200 μL
PNP Probe (in dry DMSO) Catalog Number MAK288D	200 μL
Hypoxanthine Standard (10 mM) Catalog Number MAK288E	100 μL
PNP Positive Control Catalog Number MAK288F	1 vial

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – black plates are recommended for this assay
- Fluorescence multiwell plate reader
- Protease Inhibitor Cocktail
- Dounce Homogenizer

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

PNP Assay Buffer (10×) – Make 1× buffer by adding one volume of 10× Assay Buffer to nine volumes of water. Store at –20 °C or 4 °C. Bring to 37 °C before use.

Enzyme Mix – Reconstitute with 210 µL of 1× 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 cycles.

Inosine Substrate – Aliquot and store at –20 °C. Avoid repeated freeze/thaw cycles.

PNP Positive Control – Reconstitute with 200 µL of 1× 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 cycles.

Storage/Stability
The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

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 of cold 1× PNP Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice. Transfer the tissue homogenate to a cold microfuge tube.

Transfer the tissue homogenate to a cold microfuge tube. To prepare cell extract, add 150–300 µL of cold 1× PNP Assay Buffer containing protease inhibitor cocktail (not provided) to 1–5 × 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 minutes.

Centrifuge the tissue or cell homogenate at 10,000 × *g* at 4 °C for 15 minutes. Transfer the clarified supernatant to a fresh pre-chilled tube and 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 cycles.

Hypoxanthine Standard
Dilute Hypoxanthine Standard to 1 mM by adding 10 µL of 10 mM Hypoxanthine Standard to 90 µL of 1× PNP Assay Buffer. Further dilute the Hypoxanthine Standard to 10 µM by adding 10 µL of 1 mM Hypoxanthine to 990 µL of 1× 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 pmole/well of Hypoxanthine Standard. Adjust the volume to 50 µL/well with 1× PNP Assay Buffer.

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 5× in 1× PNP Assay Buffer and add 2–4 µL of Positive Control for the assay. Make up the volume of samples and Positive Control to 50 µL/well with 1× PNP Assay Buffer. Add 50 µL of 1× PNP Assay Buffer to one well as reagent Background Control.

Notes: For unknown samples, performing a pilot experiment and testing several sample concentrations to ensure the readings are within the Standard Curve range is suggested.

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 the sample through a desalting column or by buffer exchange using a 10 kDa spin column. Use this treated sample for the assay.

Optional
Prepare a parallel sample well as sample background control to ensure the small molecules are removed by either using a desalting or spin column.

Sample Mixes
Prepare enough reagents for the number of assays to be performed. Make 50 µL of the appropriate mix for each well, see Table 1.

Table 1.
Preparation of Sample Mixes

Reagent	**Reaction Mix**	**Background Control Mix**
1× PNP Assay Buffer	45 µL	47 µL
Enzyme Mix	2 µL	2 µL
PNP Probe	1 µL	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.

Measurement
Measure fluorescence (λ_{ex} = 535 nm/λ_{em} = 587 nm) in kinetic mode for at least 30 minutes at room temperature. Choose two time points (T_1 and T_2) in the linear range (can be as short as 2 minutes) 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.

Results
Calculations
Subtract 0 Standard reading from all Standard Readings. Plot the Hypoxanthine Standard Curve. Subtract reagent background control reading from sample reading. Compare the ΔRFU [(RFU_{S2} – RFU_{BG2}) – (RFU_{S1} – RFU_{BG1})] to the Standard Curve to obtain B (pmole) 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. Removing the small molecules again using desalting column or a 10 kDa spin column if sample background control reading is higher than reagent background control is recommended.

$$\text{PNP activity} = \frac{B}{(\Delta T \times \mu g)} \times DF$$

(pmole/min/µg = µU/µg = mU/mg)

B = Hypoxanthine amount from Standard Curve (pmole)
ΔT = the reaction time (minutes)
µg = the amount of protein/well (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 µmole of hypoxanthine per minute at room temperature.

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay Not Working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	Black plates are recommended for this assay.
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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