# ab204706

# Purine Nucleoside Phosphorylase Activity Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate detection of Purine Nucleoside Phosphorylase activity.

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 4 February 2019

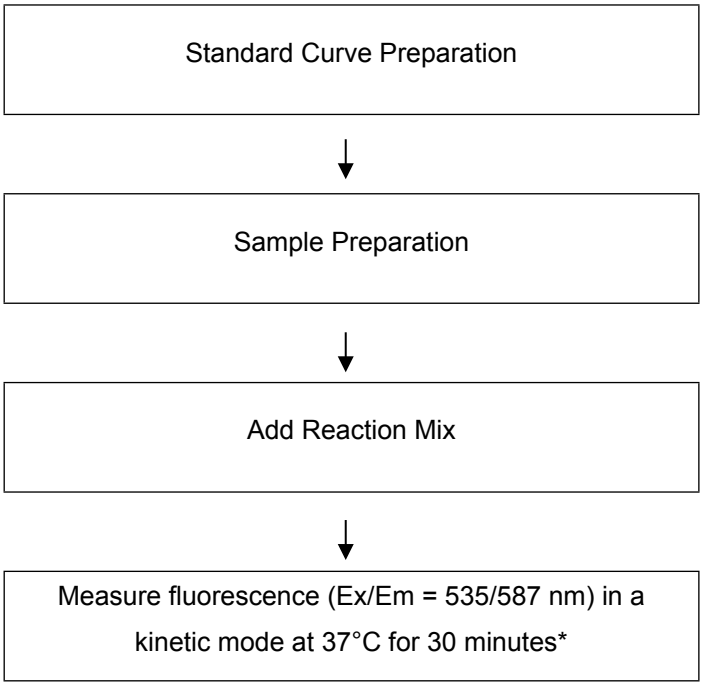
# Table of Contents

## 1. BACKGROUND

Purine Nucleoside Phosphorylase Activity Assay Kit (Fluorometric) (ab204706) is an assay where the 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. The limit of quantification is 0.5 nU recombinant Purine Nucleoside Phosphorylase.

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.

## 2. ASSAY SUMMARY




**For kinetic mode detection, incubation time given in this summary is for guidance only.*

## 3. PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

**Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

## 5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. MATERIALS SUPPLIED

| Item | Amount | Storage Condition (Before Preparation) | Storage Condition (After Preparation) |
|---|---|---|---|
| PNP Assay Buffer (10x) | 10 mL | -20°C | -20 / 4°C |
| Enzyme Mix | 1 Vial | -20°C | -20°C |
| Inosine Substrate | 200 µL | -20°C | -20°C |
| PNP Probe (in dry DMSO) | 200 µL | -20°C | -20°C |
| Hypoxanthine Standard (10 mM) | 100 µL | -20°C | -20°C |
| PNP Positive Control | 1 Vial | -20°C | -20°C |

## 7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (dd$H_2O$)
- Cold PBS
- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter Ex/Em = 535/587 nm
- 96 well plate with clear flat bottom preferably white
- Heat block or water bath
- Dounce homogenizer (if using tissue)
- (optional) 10 kD Spin Column (ab93349) or desalting column.

## 8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

## 9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **PNP Assay Buffer (10X):**

Make 1X Assay Buffer by adding one part 10X Assay Buffer to nine parts dd$H_2O$. Store at -20°C or 4°C. Warm to 37°C before use.

9.2 **Enzyme Mix:**

Reconstitute with 210 µL PNP 1X Assay Buffer and mix gently by pipetting up and down. Briefly centrifuge to collect the contents at the bottom of the tube. Aliquot enzyme mix so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

9.3 **Inosine Substrate:**

Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

9.4 **PNP Positive Control:**

Reconstitute with 200 µL PNP 1X Assay Buffer and mix gently by pipetting up and down. Briefly centrifuge to collect the contents at the bottom of the tube. Aliquot positive control so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

## 10. STANDARD PREPARATION

- Store the 10 mM solution aliquoted at -20°C
- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Prepare 100 µL of 1 mM Hypoxanthine standard by diluting 10 µL of the provided 10 mM Hypoxanthine standard with 90 µL of PNP assay buffer.

10.2 Prepare 1 mL of 10 µM Standard by diluting 10 µL of 1 mM Hypoxanthine standard with 990 µL of PNP assay buffer.

10.3 Using 10 µM Hypoxanthine standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

| Standard # | Volume of Standard (µL) | Assay Buffer (µL) | Final volume standard in well (µL) | End Conc Hypoxanthine in well (pmol/well) |
|---|---|---|---|---|
| 1 | 0 | 150 | 50 | 0 |
| 2 | 6 | 144 | 50 | 20 |
| 3 | 12 | 138 | 50 | 40 |
| 4 | 18 | 132 | 50 | 60 |
| 5 | 24 | 126 | 50 | 80 |

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

## 11. SAMPLE PREPARATION

**General Sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

### 11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1 – 5 x $10^6$ cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 150 – 300 µL of ice cold PNP Assay Buffer (containing Protease Inhibitor Cocktail).

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Incubate cells on ice for 15 – 30 minutes.

11.1.6 Transfer cell homogenate including cell debris to a cold microcentrifuge tube and agitate on a rotary shaker at 4°C for at least 15 minutes.

11.1.7 Centrifuge sample for 15 minutes at 4°C at 10,000 x g in a cold micro centrifuge to remove any insoluble material.

11.1.8 Collect supernatant and transfer to a clean pre-chilled tube and store on ice. Use lysates immediately to assay PNP activity.

11.1.9 OPTIONAL: small molecules such as xanthine, and hypoxanthine present in the samples will contribute to the

background. The molecules can be removed by passing the sample through a desalting column or by buffer exchange using a 10 kD spin column (ab93349). Use this modified sample for the assay.

11.2 **Tissue samples:**

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 100 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Suspend tissue in 300 µL of ice cold PNP Assay Buffer containing protease inhibitor cocktail.

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Incubate sample on ice for 15 – 30 minutes.

11.2.6 Centrifuge samples for 15 minutes at 4°C at 10,000 x g using a cold microcentrifuge to remove any insoluble material.

11.2.7 Collect supernatant and transfer to a clean pre-chilled tube and store on ice. Use lysates immediately to assay PNP activity.

11.2.8 OPTIONAL: small molecules such as xanthine, and hypoxanthine present in the samples will contribute to the background. The molecules can be removed by passing the sample through a desalting column or by buffer exchange using a 10 kD spin column (ab93349). Use this modified sample for the assay.

11.3 **Purified protein:**

Ready to use as supplied.

***NOTE:*** *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

## 12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, working standards, and samples as directed in the previous sections.**

12.1 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 2 – 50 µL samples (adjust volume to 50 µL/well with 1X PNP Assay Buffer).
- Background control well= 2 – 50 µL samples (adjust volume to 50 µL/well with 1X PNP Assay Buffer). ***NOTE:*** *use this background control well to ensure the small molecules are removed by the desalting or 10 kD Spin column.*
- Positive control = dilute Positive Control 5 times in 1X PNP Assay Buffer (2 µL in 8 µL 1X PNP Assay Buffer). Add 2 – 4 µL diluted Positive control to well (adjust volume to 50 µL/well with PNP Assay Buffer).

12.2 **Reaction Mix:**

Prepare 50 µL of Reaction Mix for each reaction:

| Component | Reaction Mix (µL) | Background Control Mix (µL) |
|---|---|---|
| **PNP Assay Buffer (1X)** | **45** | **47** |
| **Enzyme Mix** | **2** | **2** |
| **PNP Probe** | **1** | **1** |
| **Inosine Substrate** | **2** | **0** |

Mix enough reagents for the number of assays (samples and standards for reaction mix, background for background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number reactions +1)

12.3 Add 50 µL of Reaction Mix into each standard, sample and positive control sample wells. Mix well.

12.4 Add 50 µL of Background Reaction Mix to Background control sample well. Mix well.

12.5 Measure output on a fluorescent microplate reader at Ex/Em = 535/587 nm in a kinetic mode, every 2 – 3 minutes, for at least 30 minutes at room temperature protected from light.

***NOTE:*** *Sample incubation time can vary depending on Purine Nucleoside Phosphorylase (PNP) activity in the samples. We recommend measuring fluorescence in kinetic mode and then choosing two time points ($T_1$ and $T_2$) during the linear range.*

*RFU value at $T_2$ should not exceed the highest RFU in the standard curve. For standard curve, do not subtract $RFU_1$ from $RFU_2$ reading.*

## 13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of Hypoxanthine.

13.4 Draw the best smooth curve through these points to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Activity of PNP is calculated as:

$$\mathbf{\Delta RFU_{535/587nm} = (RFU_2 - RFU_{2BG}) - (RFU_1 - RFU_{1BG})}$$

Where:

$RFU_1$ is the sample reading at time T1.

$RFU_{1BG}$ is the background control sample at time T1.

$RFU_2$ is the sample reading at time T2.

$RFU_{2BG}$ is the background control sample at time T2.

13.6 Use the $\Delta RFU_{535/587nm}$ to obtain B pmol of Hypoxanthine generated by Purine Nucleoside Phosphorylase during the reaction time ($\Delta T = T_2 - T_1$).

13.7 Activity of PNP in the test samples is calculated as:

$$PNP\ Activity = \left(\frac{B}{\Delta T x\ \mu g\ of\ protein}\right) * D = pmol/min/mg = \mu U/\mu g = mU/$$

Where:

B = Amount of Hypoxanthine from Standard Curve (pmol).

$\Delta$T = Reaction time (minutes).

µg of protein = Original amount of protein added into the reaction well (in µg).

D = Sample dilution factor.

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

**Unit Definition:**

**1 Unit PNP activity =** amount of Purine Nucleoside Phosphorylase that hydrolyzes inosine to yield 1.0 µmol of hypoxanthine/minute at room temperature.

## 14. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

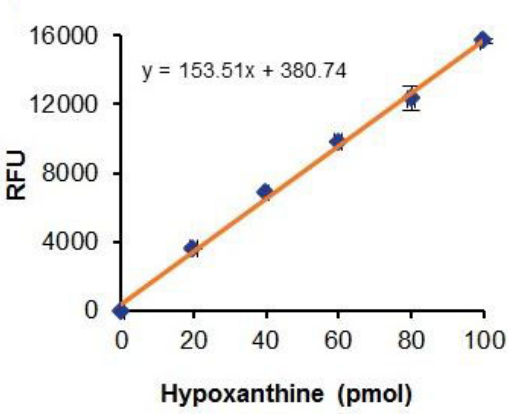



**Figure 1**. Typical Hypoxanthine Standard calibration curve.

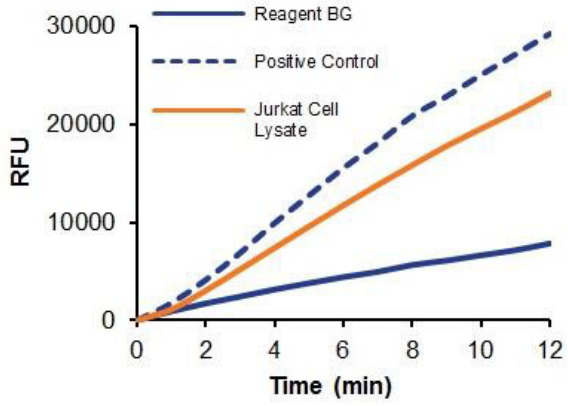



**Figure 2.** Purine Nucleoside Phosphorylase Activity in Jurkat Cell (T-lymphocyte) lysate (315 ng) and Positive Control; BG: Background.

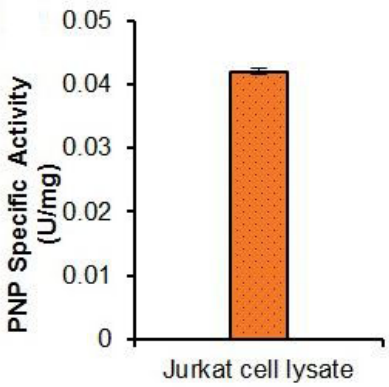



**Figure 3.** PNP specific activity in Jurkat Cell lysate.

## 15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standard, PNP probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL), samples (50 µL), positive control (50 µL) and background well (50 µL).
- Prepare PNP Reaction Mix (Number reactions + 1).

| Component | Reaction Mix (µL) | Background Reaction Mix (µL) |
|---|---|---|
| **PNP Assay Buffer (1X)** | **45** | **47** |
| **Enzyme Mix** | **2** | **2** |
| **PNP Probe** | **1** | **1** |
| **Inosine Substrate** | **2** | **0** |

- Add 50 µL of Purine Nucleoside Phosphorylase Reaction Mix to the standard, positive control and sample wells.
- Add 50 µL of Background Reaction Mix into the background sample control well. Mix well.
- Read fluorescence Ex/Em= 535/587 nm in a microplate reader in kinetic mode at room temperature for at least 30 minutes.

## 16. TROUBLESHOOTING

| Problem | Cause | Solution |
|---|---|---|
| Assay not working | Use of ice-cold buffer | Buffers must be at room temperature |
| | Plate read at incorrect wavelength | Check the wavelength and filter settings of instrument |
| | Use of a different 96-well plate | Colorimetric: Clear plates<br>Fluorometric: black wells/clear bottom plate |
| Sample with erratic readings | Samples not deproteinized (if indicated on protocol) | Use PCA precipitation protocol for deproteinization |
| | Cells/tissue samples not homogenized completely | Use Dounce homogenizer, increase number of strokes |
| | Samples used after multiple free/ thaw cycles | Aliquot and freeze samples if needed to use multiple times |
| | Use of old or inappropriately stored samples | Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use |
| | Presence of interfering substance in the sample | Check protocol for interfering substances; deproteinize samples |
| Lower/ Higher readings in samples and Standards | Improperly thawed components | Thaw all components completely and mix gently before use |
| | Allowing reagents to sit for extended times on ice | Always thaw and prepare fresh reaction mix before use |
| | Incorrect incubation times or temperatures | Verify correct incubation times and temperatures in protocol |

| Problem | Cause | Solution |
|---|---|---|
| Standard readings do not follow a linear pattern | Pipetting errors in standard or reaction mix | Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible |
| | Air bubbles formed in well | Pipette gently against the wall of the tubes |
| | Standard stock is at incorrect concentration | Always refer to dilutions on protocol |
| Unanticipated results | Measured at incorrect wavelength | Check equipment and filter setting |
| | Samples contain interfering substances | Troubleshoot if it interferes with the kit |
| | Sample readings above/ below the linear range | Concentrate/ Dilute sample so it is within the linear range |

## 17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure.

- RIPA buffer – it contains SDS which can destroy/decrease the activity of the enzyme.
- Small molecules such as xanthine and hypoxanthine – can cause background signal.

## 18.FAQ

## 19.NOTES

**UK, EU and ROW**
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