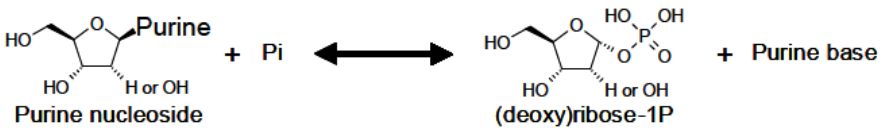
PRECICE® PNP Assay Kit

Purine Nucleoside Phosphorylase Assay Kit

For research use only. Not for use in diagnostic procedures

I. Background

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) is involved in salvage pathway of the purine metabolism. In the presence of inorganic phosphate PNP catalyzes the cleavage of the glycosidic bond of ribo- or deoxyribonucleosides (inosine, guanosine or their deoxyanalogues) to generate the purine base and ribose- or deoxyribose-1-phosphate. The reaction is reversible for natural substrates:



Purine nucleoside phosphorylase deficiency is a rare autosomal recessive metabolic disorder which results in accumulation of toxic metabolites in T-cell lymphocytes and severe immunodeficiency. In addition, purine nucleoside phosphorylase deficiency is associated with neurologic symptoms, including mental retardation and muscle spasticity, and increased risk of autoimmune disorders.

Since most parasitic protozoan are obligate auxotrophs of purines and entirely depend therefore on their purine salvage pathways, PNP of apicomplexan parasites (*P. falciparum* and *T. gondii*) is currently explored as a potential target for drug development.

II. Direct assay for PNP activity

PRECICE® PNP Assay Kit provides an enzymatic tool allowing direct continuous spectrophotometric monitoring of PNP activity in a convenient 96-well plate format. In the assay, PNP activity is measured as a rate of production of hypoxanthine, which is directly oxidized by XDH enzyme with concomitant reduction of NAD^+ to NADH measurable by absorbance at 340nm (Fig. 1).

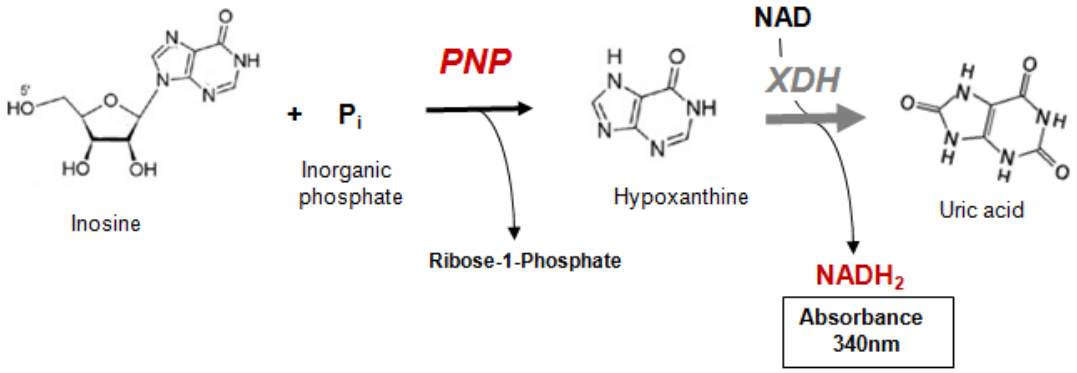


Figure 1. Enzymatic principle of PRECICE® PNP Assay Kit.

The assay is developed for measuring PNP activity *in vitro* or in cell lysates.

For maximal accuracy, the assays with cell lysates are run **with and without inosine** in parallel. The absorbance rate observed in the absence of inosine is used as blank and is subtracted from the absorbance rate measured in its presence.

III. Equipments required

1) Plate agitator
2) Plate reader fitted with a filter 340nm (ex. Labsystems iEMS Reader MF (Thermo), Epoch (BioTec); PerkinElmer.

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IV. Kit Contents for 24 samples:

Once dissolved, the reagents provided in the kit are not stable and should be stored on ice and used the day of preparation. The kit allows to perform 24 analysis in a time (8 samples in triplicate, 12 samples in duplicate).

A standard PRECICE® PNP Assay Kit contains:

- one 2mL-tube **"Cofactor 1"** (DTT);
- one 2mL-tube **"Cofactor 2"** (NAD);
- one vial "**XDH Enzyme**";
- one 15mL tube "**Blank"** (pre-filled with 10mL of "Reaction buffer");
- one 15mL tube **"Reaction mixture"** (pre-filled with 50µL of 100mM inosine);
- one tube of "**Purified PNP**" (human recombinant) to be used as a positive control;
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797

IMPORTANT:
The following instructions are given to measure the activity of PNP enzyme, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® PNP Assay Kit or of one or several of its components, in other conditions than those described in this user manual and/or for other purpose than R&D.

V. Preparation of 10ml "Reaction mixture"

1. Add 500µL of deionized water to the tube with "**Cofactor 1"** to prepare 0.1M DTT. Agitate until complete dissolution of the powder. Transfer dissolved "Cofactor 1" to the tube with **"XDH enzyme"**. Agitate gently until complete dissolution of the powder

2. Quantitatively transfer the content of the tubes with **"Cofactor 2**" and solubilized **"XDH enzyme"** to a 15-ml tube **"Blank"**.

To do so:

-pipet 1ml of buffer from **"Blank"** to each of 2 tubes and mix them by inverting or pipeting up and down until the powder dissolved.

- transfer by pipeting the content of two **tubes** back into a vial **"Blank"**.

- repeat to be sure that all reagent and enzymes of the small tubes and vial are recovered. mix by gently inverting until complete dissolution. Avoid bubbles.

4. Transfer 5mLof "Blank" solution to **"Reaction mixture"** tube pre-filled with inosine
You have prepared: 5ml of **"Blank"**
5ml of **"Reaction mixture"** containing **1mM inosine.**
Preheat both mixtures at 37°C for 15 mintues.

VI. Microplate preparation

1. **Preparation of hemolysates.** The pellet of PBS-washed erythrocytes from 100µL of blood was frozen-thawed twice, resuspended in 2mL of ice-cold deionized water and used directly for PNP quantification.

2. **Positive control.** Add indicated volume of deionized water (1.8mL) to **"Purified PNP"** enzyme (human recombinant, 1.3Units, provided lyophilized) to obtain solution at 85 µmol/h/mL and mix gently until the powder is dissolved. Once dissolved, PNP should be used immediately. Add 4µL of resuspended PNP enzyme per well in line A as shown below:

2. Add 4µL of hemolysates (indicated as S1-S11) per well as shown next page:

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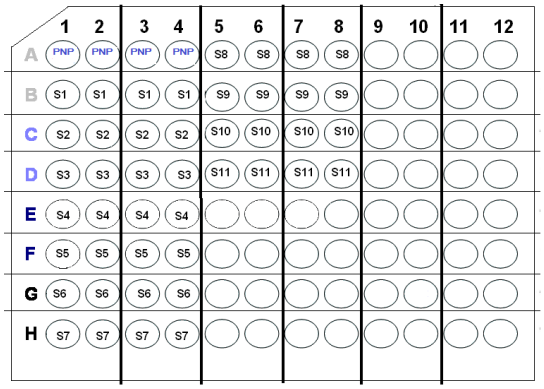

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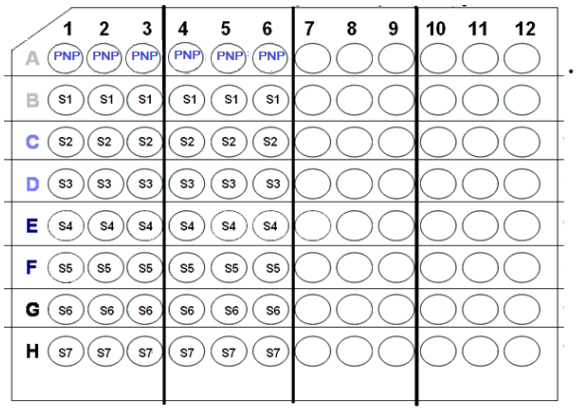

3. Add 200µL of "Blank" per well and 200µL of **"Reaction mixture"** containing **1mM inosine** as shown below:

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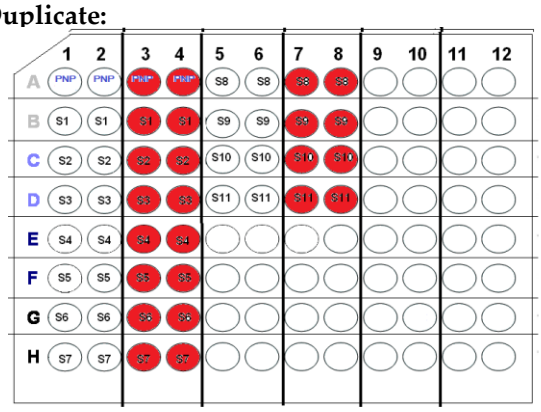

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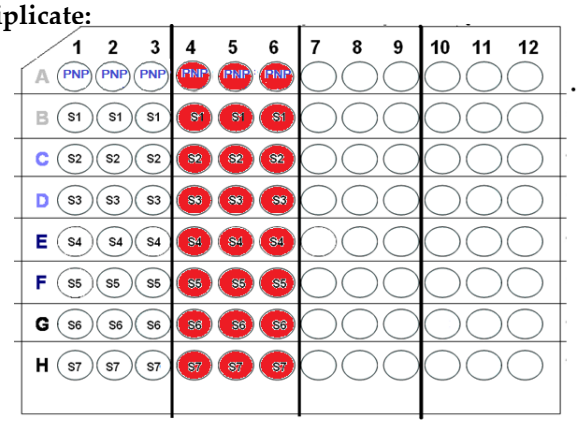

4. Program plate reader for kinetics absorbance reading (every 1 min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 20min with data collection every 1min. Typical results obtained with purified human recombinant PNP and RBC lysates are shown on Table 1 / Figure 1 and Table 2/ Figure 2, respectively.

A. Calculation of activity of human recombinant PNP (positive control)

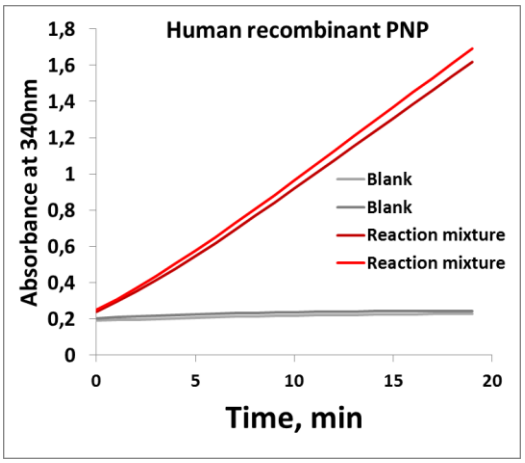

Figure 1. Kinetics of inosine hydrolysis by human recombinant PNP in the absence and the presence of inosine. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797).

Table 1.				
Time, min	Blank		Reaction mixture	
0	0,19	0,204	0,241	0,251
1	0,194	0,209	0,294	0,308
2	0,197	0,214	0,352	0,369
3	0,2	0,218	0,414	0,435
4	0,203	0,222	0,478	0,504
5	0,206	0,226	0,546	0,576
6	0,209	0,23	0,618	0,65
7	0,212	0,232	0,692	0,726
8	0,215	0,233	0,767	0,804
9	0,217	0,235	0,842	0,883
10	0,219	0,237	0,918	0,964
11	0,22	0,238	0,996	1,045
12	0,222	0,24	1,074	1,125
13	0,223	0,241	1,152	1,207
14	0,224	0,242	1,231	1,289
15	0,225	0,243	1,309	1,371
16	0,226	0,244	1,387	1,452
17	0,227	0,244	1,464	1,531
18	0,228	0,245	1,542	1,612
19	0,229	0,245	1,619	1,691
20	0,23	0,246	1,695	1,769
Absorbance rate per min	0,002	0,002	0,074	0,078
Absorbance per hour	0,116	0,116	4,457	4,653
PNP activity, nmol/h/mL			885,910	925,937

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1. Calculate the absorbance rate per hour for reaction buffers with **inosine** (AR_{INO}) and without (AR_{blanc}).

2. Calculate Mean AR_{INO} and Mean AR_{blank}

3. Calculate PNP activity as follows:

$$\text{PNP Activity (in nmol /ml/ hour)} = \frac{AR_{INO} - AR_{blank}}{4.9} \times 10^3 = \frac{(4.555 - 0.116)}{4.9} \times 10^3 = \mathbf{905.9\ nmol/\ ml/\ h}$$

where: Mean AR_{INO} = 4.555

Mean AR_{blank} = 0.116

4.9 is the absorbance of 1mM NADH at 340nm in 200µL- round-bottom well of 96-well microplate (Corning, Costar®, ref. 3797, provided).

B. Calculation of PNP activity in hemolysates

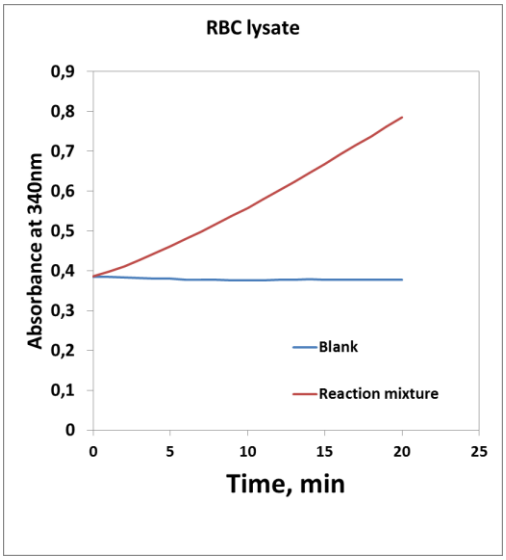

Figure 2. Kinetics of inosine hydrolysis by PNP in hemolysates in the absence and the presence of inosine. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797).

Time, min	Blank			Reaction mixture		
0	0,385	0,364	0,372	0,386	0,384	0,375
1	0,385	0,363	0,37	0,398	0,396	0,386
2	0,384	0,365	0,369	0,411	0,41	0,397
3	0,382	0,363	0,369	0,427	0,427	0,412
4	0,381	0,362	0,368	0,444	0,443	0,427
5	0,38	0,361	0,367	0,462	0,459	0,444
6	0,377	0,36	0,367	0,48	0,477	0,461
7	0,377	0,359	0,366	0,498	0,495	0,479
8	0,377	0,358	0,365	0,518	0,514	0,497
9	0,376	0,358	0,365	0,538	0,533	0,516
10	0,376	0,357	0,364	0,558	0,553	0,536
11	0,376	0,357	0,364	0,579	0,574	0,555
12	0,377	0,356	0,363	0,601	0,595	0,576
13	0,378	0,356	0,363	0,623	0,617	0,597
14	0,379	0,356	0,364	0,646	0,639	0,618
15	0,378	0,356	0,364	0,668	0,661	0,639
16	0,378	0,357	0,364	0,692	0,684	0,661
17	0,378	0,357	0,364	0,715	0,706	0,683
18	0,378	0,357	0,364	0,738	0,729	0,705
19	0,378	0,357	0,364	0,762	0,752	0,727
20	0,378	0,357	0,365	0,786	0,775	0,749
Absorbance rate per min	0,000	0,000	0,000	0,020	0,020	0,019
Absorbance per hour	-0,018	-0,025	-0,021	1,221	1,192	1,148
PNP activity, nmol/h/mL				252,82	248,19	238,41

1. For first two hours, calculate the absorbance rate per hour for reaction buffers with inosine (AR_{INO}) and without (AR_{blanc}). Calculate Mean AR_{INO} and Mean AR_{blank}

2. Measure the concentration of hemoglobin [Hgb] in sonicated hemolysates using Drabkin's reagent and calculate final [Hgb] concentration used in assay.

3. PNP activity is calculated by the following formula:

$$\text{Activity} = \frac{\text{Mean } AR_{INO} - \text{Mean } AR_{blank}}{4.9 \times \mathbf{[Hgb]}} \times 10^3 = \frac{(1.187 + 0.021)}{4.9 \times \mathbf{0.24}} \times 10^3 = \mathbf{1027\ nmol/\ hour\ /\ mg\ of\ Hgb}$$

where: Mean AR_{INO} = 1.190

Mean AR_{blank} = -0.021

[Hgb], final haemoglobin concentration used in assay **= 0.24**mg/ml

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