PRECICE® HPRT Assay Kit

Hypoxanthine-guanine phosphoribosyltransferase Assay Kit

For research use only. Not for use in diagnostic procedures

I. Background

Hypoxanthine phosphoribosyltransferase is a purine salvage enzyme that catalyzes the reversible transfer of the 5-phosphoribosyl moiety from α–D-5-phosphoribosyl-1-pyrophosphate (PRPP) to a purine base (hypoxanthine or guanine) to form a nucleoside monophosphate (inosine monophosphate or guanosine monophosphate, respectively). In the presence of pyrophosphate, HPRT enzyme catalyzes also the hydrolysis of IMP and GMP, although this reverse reaction is much less favored than forward one. Human HPRT enzyme does not hydrolyse XMP.

HPRT1 gene is one of the best characterized in the human genome for two reasons: (i) *HPRT1* gene is widely used as a somatic cell genetic marker in genotoxicity / mutagenicity studies; (ii) the defects within the human enzyme are associated with inherited gouty arthritis and Lesch–Nyhan syndrome and more than 300 disease-associated mutations in human *HPRT1* gene leading to partial or complete deficiencies of the HPRT enzyme have been described[1]. In view of the high variability of HPRT1 gene, a rapid biochemical assay would be useful both for basic science and clinical research.

In addition, since most parasitic protozoan are obligate auxotrophs of purines and entirely depend therefore on their purine salvage pathways, protozoan HPRT enzyme is an attractive target for the discovery of new anti-parasitic drugs[2]. The enzymatic microplate assay enabling monitoring of HPRT activity may therefore accelerate the search of new anti-parasitic drugs.

II. Principle

PRECICE® HPRT Assay Kit provides an enzymatic tool for continuous spectrophotometric monitoring of HPRT activity in a convenient 96-well plate format. In the assay, HPRT activity is measured as a rate of production of IMP, which is oxidized by recombinant IMPDH enzyme with simultaneous reduction of NAD^+ to NADH measurable by absorbance at 340nm (Fig. 1).

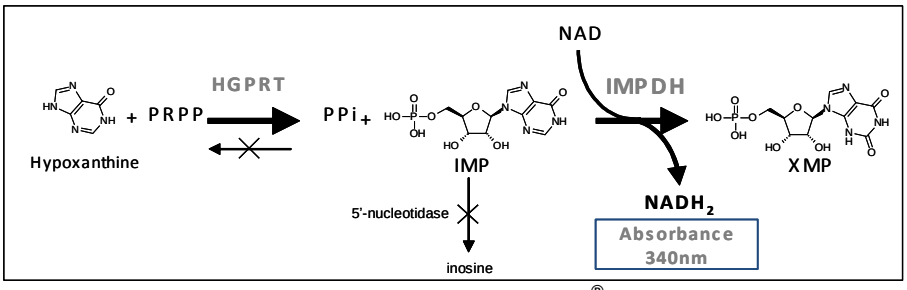

Figure 1. Enzymatic principle of PRECICE® HPRT Assay Kit.

The assay is developed for measuring HPRT activity *in vitro* or in lysates of cells.

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

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 48 analysis (24 samples in duplicate):

Once dissolved, the reagents provided in the kit are not stable and should be stored on ice and used the day of preparation. For practical reasons, the kit is prepared of two identical sets of tubes to allow to perform only 24 analysis in a time (8 samples in triplicate, 12 samples in duplicate).

A standard PRECICE® HPRT Assay Kit contains 2 sets of tubes, each set contains:

- one tube **"Cofactor 1"** (**DTT);**
- one tube **"Cofactor 2"** (**NAD);**
- one tube **"Bacterial IMPDH" ;**
- one tube **"Reaction buffer 10x" (1mL);**
- one tube of "**Human Recombinant HPRT**" for preparing enzyme solution at 75mU/ml (94.6nmol/h/ml);
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797

Not provided:

PRPP (α–D-5-phosphoribosyl-1-pyrophosphate, available at Sigma-Aldrich, ref. P8296)

***Important:* PRPP is highly unstable once dissolved. We recommend to prepare the tubes with inducated mg of PRPP, store them as a powder at -20°C and dissolve it at very last moment.**

IMPORTANT:
The following instructions are given to measure the activity of HPRT enzyme, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® HPRT 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 hemolysates

This protocol was developed with erythrocytes purified from 1mL of peripheral blood using Ficoll-Hypaque gradient and washed once with PBS.

The pellet of PBS-washed packed erythrocytes (from 1ml of blood) was resuspended in 4mL of ice-cold dH_20 and sonicated for 1min on ice (Sonopuls, Bandelin, 20% cycle, 50% power). The sonicated hemolysates were immediately used for HPRT measurement without additional centrifugation.

The hemolysates can be also prepared by numerous freeze-thawing of erythrocytes resuspended in water and high speed centrifugation. Since the efficiency of hemolysis and release of HPRT enzyme depends on the method used for RBC disruption, we recommend to use always the same protocol of hemolysate preparation.

IV. Preparation of 10ml "Reaction mixture 1x" (for performing 48 assays of 200µL, 24 with and 24 without PRPP)

1. Add 250µL of of deionized water to the tube with "Recombinant IMPDH". Agitate gently until complete dissolution of the powder.

2. Label a clean 15-ml tube **"Reaction mixture 1x"**, transfer 1ml of **"Reaction buffer 10x",** followed by addition of 9 ml of deionized water.

3. Add the content of 2 tubes with "**Cofactor 1", Cofactor 2"** and **"Bacterial IMPDH"** to a 15-ml tube **"Reaction mixture 1x"**.

To do so:

-pipet 1ml of buffer from **"Reaction mixture 1x"** 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 **"Reaction mixture 1x"**.

- 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.

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4. Weight 5mg of PRPP (Sigma-Aldrich, ref. P8296) in a clean labeled tube (15ml), add 5ml of prepared **"Reaction mixture 1x".**

You have prepared: 5ml of **"Reaction mixture 1x" (without PRPP, Blank)**
5ml of **"Reaction mixture 1x" with 2mM PRPP**

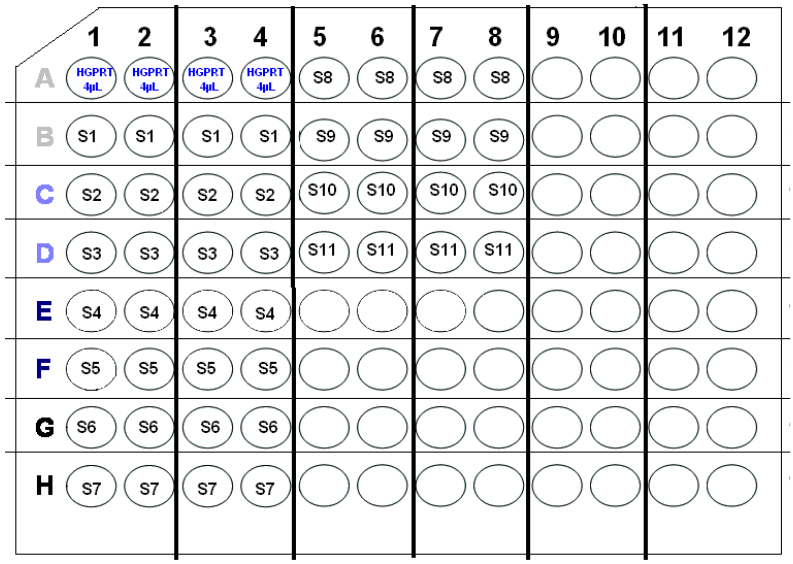
V. Microplate preparation (duplicate, 12 samples)

1. **Positive control.** Add indicated volume of deionized water to lyophilized human recombinant HPRT enzyme to provide 75mU/ml solution and mix gently until the powder is dissolved. Add 4µL of HPRT enzyme per well in line A as shown below :

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

Since the hemolysates show inherent optical density (OD) at 340nm, we strongly recommend to check the initial density of diluted hemolysates at 340nm before starting HPRT quantification. To do it, add 2, 4, or 6µL of hemolysates to the wells of 96-well plate followed by the addition of deionized water (qsp 200µL). Agitate for 2min and read the absorbance at 340nm. Use the volume of hemolysates providing OD in the range from 0.9 to 1.1 (usually it corresponds to 4µL of hemolysates per well).

Duplicate:



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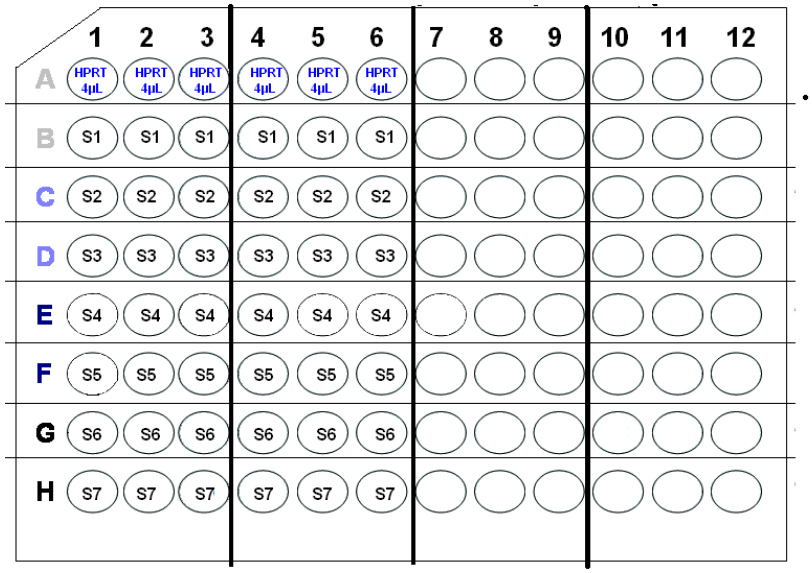

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3. Add 200µL of **"Reaction mixture 1x without PRPP" (Blank)** per well and 200µL of **"Reaction mixture 1x" with 2mM PRPP** as shown below:

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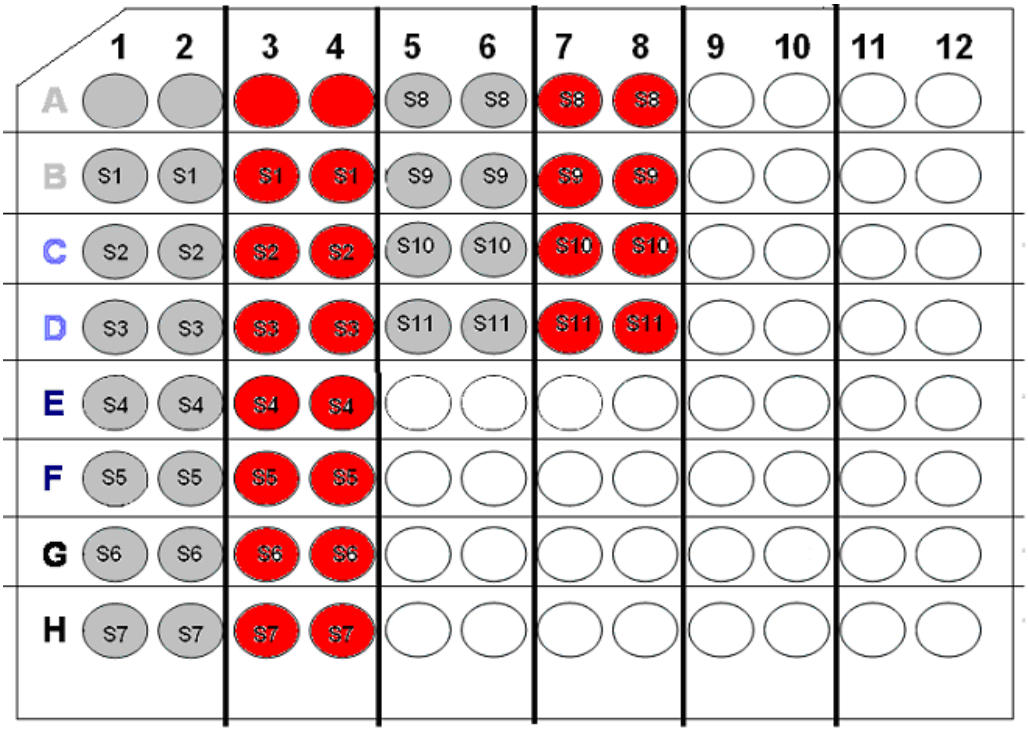
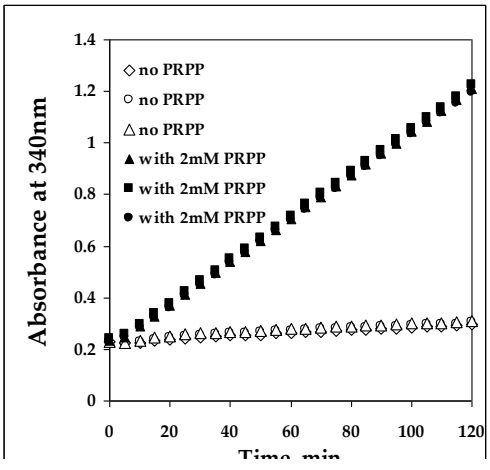


4. Program plate reader for kinetics absorbance reading (every 5 min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 2min and monitor the reaction at 340nm at 37°C for 2h with data collection every 5min.

Typical results obtained with PRPP added in the presence of 1mM Hx or its absence are shown on Table 1 and Figure 1.

Table 1.



Time, min	Absorbance at 340nm without PRPP			Absorbance at 340nm with 2mM PRPP		
0	0.228	0.225	0.227	0.232	0.24	0.227
5	0.224	0.222	0.225	0.248	0.258	0.244
10	0.226	0.228	0.235	0.287	0.298	0.283
15	0.232	0.239	0.245	0.328	0.338	0.323
20	0.237	0.245	0.251	0.369	0.378	0.362
25	0.241	0.251	0.257	0.413	0.424	0.407
30	0.247	0.255	0.261	0.455	0.465	0.449
35	0.249	0.258	0.264	0.495	0.506	0.49
40	0.253	0.261	0.268	0.539	0.55	0.533
45	0.253	0.262	0.268	0.577	0.588	0.571
50	0.256	0.266	0.272	0.621	0.634	0.616
55	0.261	0.27	0.276	0.663	0.675	0.657

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Figure 1. Time course of IMP formation by human recombinant HPRT (1.5mU/ml) incubated in the presence of PRPP in standard reaction buffer or its absence. After vigorous shaking for 2min, 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).

60	0.264	0.272	0.278	**0.705**	**0.718**	**0.699**
65	0.267	0.275	0.282	**0.749**	**0.763**	**0.743**
70	0.269	0.278	0.284	**0.791**	**0.804**	**0.783**
75	0.272	0.281	0.287	**0.833**	**0.846**	**0.825**
80	0.275	0.284	0.29	**0.875**	**0.89**	**0.868**
85	0.277	0.286	0.292	**0.917**	**0.931**	**0.91**
90	0.279	0.288	0.294	**0.957**	**0.972**	**0.949**
95	0.281	0.29	0.296	**0.999**	**1.015**	**0.992**
100	0.285	0.294	0.3	**1.042**	**1.058**	**1.034**
105	0.289	0.296	0.302	**1.083**	**1.099**	**1.076**
110	0.289	0.297	0.303	**1.123**	**1.139**	**1.115**
115	0.291	0.299	0.306	**1.165**	**1.181**	**1.156**
120	0.296	0.304	0.31	**1.207**	**1.224**	**1.198**
Absorbance rate per hour	0.010	0.013	0.036	0.376	0.366	**0.497**

Table 2.

VI. Calculation of activity of recombinant HPRT

1. For first two hours, calculate the absorbance rate per hour for reaction buffers with 2mM PRPP (AR_{PRPP}) and without PRPP (AR_{blanc}).

2. Calculate Mean $\mathbf{AR_{PRPP}}$ and Mean $\mathbf{AR_{blank}}$

3. Calculate HPRT activity as follows:

$$\text{HPRT Activity (in nmol /ml/ hour)} = \frac{\mathbf{AR_{PRPP} - AR_{blank}}}{\varepsilon \,.\, l} \times 10^6 = \frac{0.500 - 0.037}{6220.\ 0.789} \times 10^6 = \mathbf{94.6 nmol/\ ml/\ h}$$

where: ε is the molar extinction coefficient of NADH at 340nm : ε = 6220 $M^{-1}.cm^{-1}$
l is the path-length = 0.789 for a 200µL- round-bottom well of 96-well microplate (Corning, Costar®, ref. 3797)

3. Calculation of HPRT activity in hemolysates

5.1. For first two hours, calculate the absorbance rate per hour for reaction buffers with 2mM PRPP (AR_{PRPP}) and without PRPP (AR_{blanc}). Calculate Mean $\mathbf{AR_{PRPP}}$ and Mean $\mathbf{AR_{blank}}$

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

5.3. HPRT activity is calculated by the following formula:

$$\text{Activity (in nmol / mg of hemoglobine per hour)} = \frac{\text{Mean } \mathbf{AR_{PRPP}} - \text{Mean } \mathbf{AR_{blank}}}{\varepsilon \,.\, l \,.\, \mathbf{[Hgb]}} \times 10^6 = \mathbf{84.62\ nmol/\ mg\ of\ Hgb\ /\ h}$$

where: Mean $\mathbf{AR_{PRPP}}$ = 0.490
Mean $\mathbf{AR_{blank}}$ = 0.033
ε is the molar extinction coefficient of NADH at 340nm : ε = 6220 $M^{-1}.cm^{-1}$
l is the path-length: l=0.789 for a 200µL round-bottom well of 96-well microplate (Corning, Costar®, ref. 3797)
[Hgb], final haemoglobin concentration used in assay **=** 1.1mg/ml

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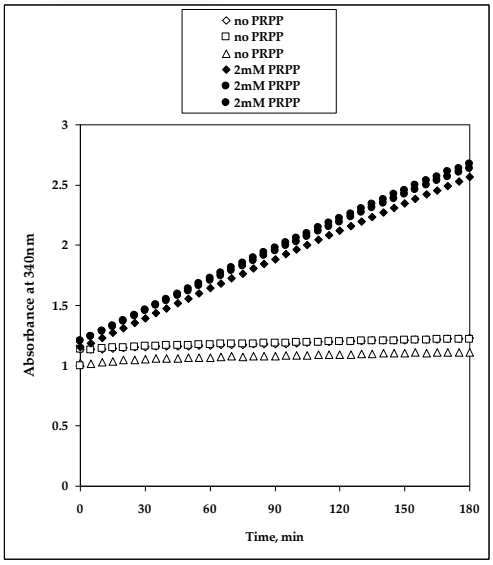

Figure 2. Kinetics of IMP formation in hemolysates incubated in the presence of PRPP (Sigma-Aldrich, ref. P8296) in standard reaction buffer. After vigorous shaking for 2min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797).

Time, min	no PRPP			no PRPP		
0	1.139	1.139	1.02	1.156	1.205	1.205
5	1.135	1.132	1.019	1.186	1.242	1.24
10	1.138	1.141	1.028	1.23	1.289	1.284
15	1.143	1.146	1.037	1.271	1.332	1.326
20	1.149	1.15	1.046	1.311	1.375	1.369
25	1.153	1.156	1.051	1.353	1.42	1.411
30	1.156	1.16	1.055	1.396	1.464	1.453
35	1.159	1.163	1.058	1.436	1.508	1.498
40	1.16	1.166	1.061	1.477	1.551	1.539
45	1.162	1.167	1.063	1.517	1.593	1.58
50	1.164	1.169	1.064	1.559	1.638	1.622
55	1.168	1.174	1.07	1.603	1.683	1.665
60	1.168	1.174	1.07	1.642	1.724	1.704
65	1.171	1.178	1.074	1.685	1.769	1.746
70	1.173	1.18	1.077	1.726	1.813	1.79
75	1.174	1.18	1.075	1.763	1.851	1.827
80	1.177	1.183	1.079	1.806	1.896	1.873
85	1.178	1.186	1.082	1.846	1.938	1.913
90	1.179	1.187	1.082	1.885	1.977	1.954
95	1.181	1.188	1.084	1.926	2.022	1.995
100	1.184	1.191	1.086	1.963	2.059	2.03
105	1.194	1.191	1.085	2.002	2.099	2.071
110	1.199	1.195	1.091	2.044	2.145	2.116
115	1.201	1.196	1.092	2.083	2.183	2.155
120	1.203	1.197	1.091	2.119	2.221	2.19
Absorbance per hour	*0.031*	*0.030*	*0.034*	***0.487***	***0.508***	***0.497***

References:

[1] Torres, R. J.,Puig, J. G., Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency: Lesch-Nyhan syndrom. *Orphanet J Rare Dis.* **2**, 48-57 (2007).

[2] Datta, A.K, Datta, R., Sen, B. Antiparasitic chemotherapy: tinkering with the purine salvage pathway. *Adv. Exp. Med. Biol.* **625**, 116-132 (2008).

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