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).

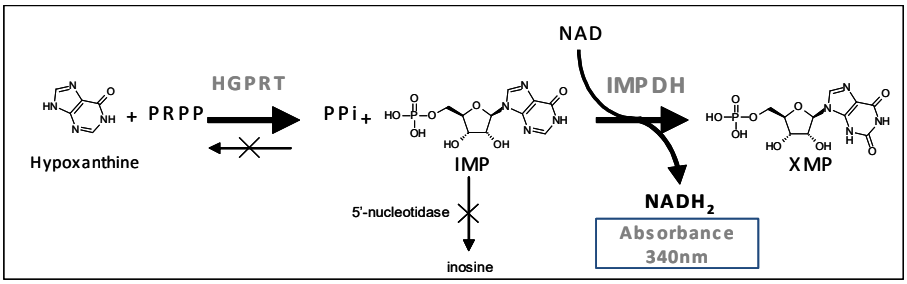


Figure 1. Enzymatic principle of PRECICE® HPRT Assay Kit.

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

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.

IV. Kit Contents for 24 analysis:

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

A standard PRECICE® HPRT Assay Kit contains:

- one tube **"Cofactor 1" (cysteine);**
- one tube **"Cofactor 2" (NAD);**
- one tube "**Highly active IMPDH" ;**
- one tube "**Reaction buffer 10x" (1mL, contains hypoxanthine);**
- 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, CAS 108321-05-7available at Santa Cruz Biotechnology, ref. sc-217240)

***Important:* PRPP is highly unstable once dissolved. We recommend to prepare the tubes with indicated 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"

1. Add 9ml (corresponding to 9g) of deionized water to provided 15-mL tube containing "Reaction Buffer 10x" to prepare "Reaction Buffer 1x";

2. Transfer the contents of the tubes ("Cofactor 1", "Cofactor 2", powder) into a tube with "Reaction buffer 1x" by adding 1 mL of "Reaction buffer 1x" to each tube, close, vortex, spin shortly to recover all liquid to the tube and transfer the content back into the tube with "Reaction buffer 1x";

3. Solubilize "Highly active IMPDH" by adding 1ml of "Reaction buffer 1x" with co-factors 1 and 2. Close, agitate and transfer the content tube back into a vial "Reaction buffer 1x".

4. Weight 5mg of PRPP (SCBT Ref sc-21740 or Sigma-Aldrich, ref. P8296) in a clean labeled tube (15ml), add 5ml of prepared **"Reaction buffer 1x".**

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

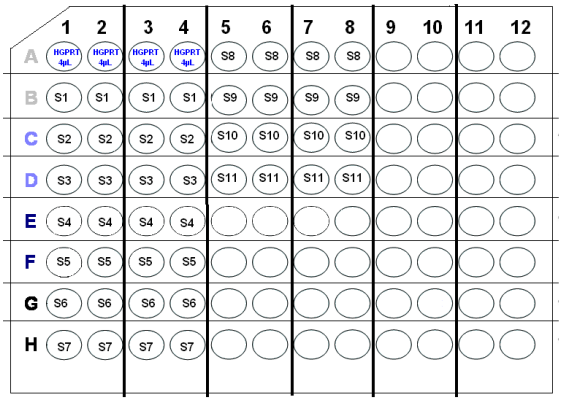
V. Microplate preparation (duplicate, 12 samples)

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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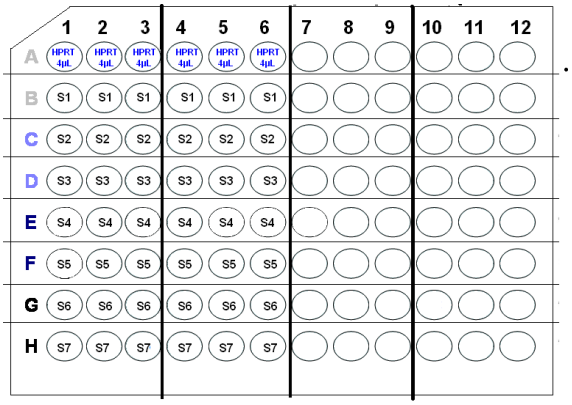
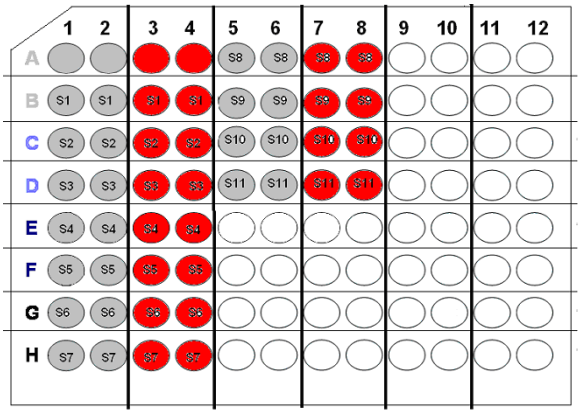


3. Add 200µL of **"Reaction mixture 1x without PRPP" (Blank)** per well and 200µL of **"Reaction mixture 1x" with 2mM PRPP** as shown below:

Duplicate:



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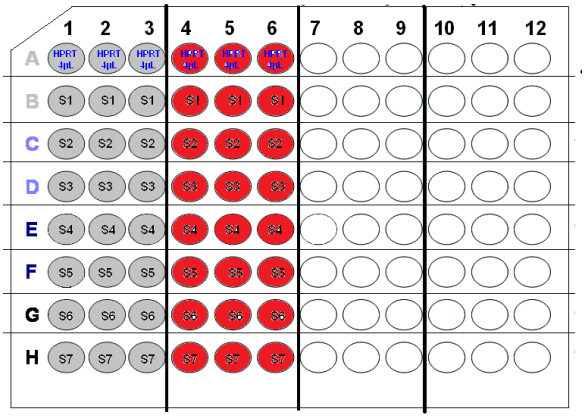

4. Program plate reader for kinetics absorbance reading (every 2 min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 2min and monitor the reaction at 340nm at 37°C for 1h with data collection every 2min.

Typical results obtained in the presence of PRPP or in its absence are shown on Table 1 and Figure 1 (for recombinant HGPRT) and Table 2 and Figure 2 (for hemolysates).

VI. Calculation of activity of recombinant HPRT

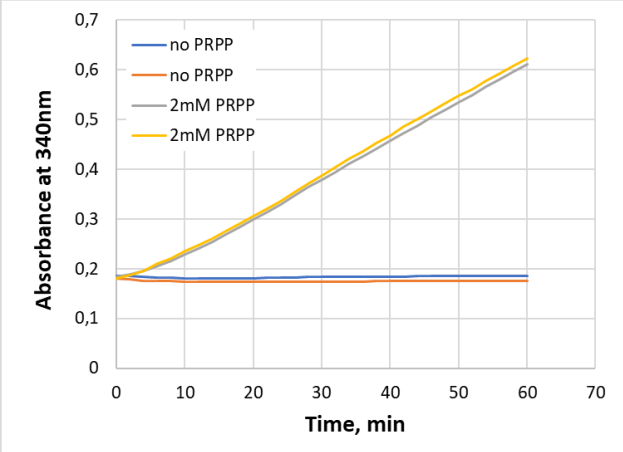

Figure 1. Time course of IMP formation by human recombinant HPRT (1.5mU/ml) incubated in the presence of PRPP in reaction buffer or its absence. After vigorous shaking, the absorbance at 340nm was monitored at 37°C using iEMS microplate reader (Thermo) and round-bottom 96-well microplate (Corning® ref 3797).

Table 1.

Time, min	Absorbance at 340nm without PRPP		Absorbance at 340nm with 2mM PRPP	
0	0,185	0,18	0,184	0,18
2	0,185	0,178	0,188	0,187
4	0,183	0,176	0,196	0,196
6	0,182	0,176	0,206	0,211
8	0,182	0,175	0,215	0,22
10	0,181	0,174	0,228	0,235
12	0,181	0,174	0,241	0,247
14	0,181	0,174	0,253	0,261
16	0,181	0,174	0,268	0,275
18	0,181	0,173	0,283	0,29
20	0,181	0,174	0,298	0,305
22	0,182	0,173	0,314	0,321
24	0,182	0,174	0,329	0,336
26	0,182	0,174	0,347	0,353
28	0,183	0,174	0,363	0,37
30	0,183	0,174	0,378	0,387
32	0,183	0,174	0,394	0,404
34	0,183	0,174	0,41	0,42
36	0,184	0,174	0,425	0,436
38	0,184	0,175	0,441	0,453
40	0,184	0,175	0,458	0,468
42	0,184	0,175	0,473	0,485
44	0,185	0,175	0,488	0,5
46	0,185	0,175	0,504	0,515
48	0,185	0,176	0,519	0,532
50	0,185	0,176	0,534	0,547
52	0,186	0,176	0,549	0,561
54	0,186	0,176	0,565	0,577
56	0,186	0,176	0,58	0,592
58	0,186	0,176	0,595	0,607
60	0,186	0,176	0,61	0,623
Mean AR (AU/h),	**AR_{blank}** 0,002		**AR_{PRPP}** 0,454	

1. Calculate the absorbance rate per hour for reaction buffers with 2mM PRPP (AR_{PRPP}) and without PRPP (AR_{blanc}).

2. Calculate Mean **AR_{PRPP}** and Mean **AR_{blank}**

3. Calculate HPRT activity as follows:

$$\text{HPRT Activity} = \frac{AR_{PRPP} - AR_{blank}}{\varepsilon \cdot l} \times 10^6 = \frac{0.454 - 0.002}{6220 \cdot 0.772} \times 10^6 = 94.2 \text{nmol/ ml/ h}$$

where: ε is the molar extinction coefficient of NADH at 340nm : ε = 6220 $M^{-1}.cm^{-1}$

l is the path-length = 0.772 for a 200µL- round-bottom well of 96-well microplate (Corning, Costar®, ref. 3797)

VII. Calculation of HPRT activity in hemolysates

1. Calculate the absorbance rate per hour for reaction buffers with 2mM PRPP (AR_{PRPP}) and without PRPP (AR_{blanc}). Calculate Mean **AR_{PRPP}** and Mean **AR_{blank}**.

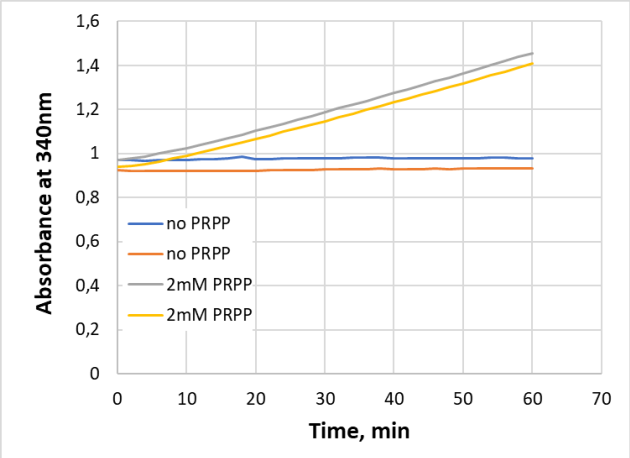

Figure 1. Time course of IMP formation in human hemolyastes incubated in the presence of PRPP in reaction buffer or its absence. After vigorous shaking, the absorbance at 340nm was monitored at 37°C using iEMS microplate reader (Thermo) and round-bottom 96-well microplate (Corning® ref 3797).

Time, min	Absorbance at 340nm without PRPP		Absorbance at 340nm with 2mM PRPP	
0	0,971	0,924	0,97	0,938
2	0,97	0,921	0,978	0,945
4	0,968	0,919	0,987	0,953
6	0,969	0,919	0,999	0,964
8	0,971	0,919	1,012	0,977
10	0,972	0,919	1,025	0,991
12	0,974	0,92	1,04	1,005
14	0,974	0,92	1,054	1,019
16	0,978	0,921	1,07	1,034
18	0,984	0,922	1,086	1,05
20	0,975	0,922	1,102	1,066
22	0,975	0,923	1,119	1,081
24	0,977	0,925	1,136	1,098
26	0,976	0,924	1,152	1,114
28	0,977	0,925	1,169	1,13
30	0,979	0,927	1,186	1,147
32	0,978	0,927	1,205	1,164
34	0,98	0,928	1,221	1,181
36	0,981	0,929	1,238	1,198
38	0,983	0,931	1,257	1,216
40	0,978	0,93	1,274	1,232
42	0,978	0,93	1,291	1,25
44	0,979	0,93	1,31	1,267
46	0,979	0,931	1,328	1,284
48	0,977	0,93	1,345	1,301
50	0,979	0,931	1,363	1,319
52	0,979	0,932	1,382	1,337
54	0,98	0,934	1,401	1,354
56	0,98	0,934	1,419	1,372
58	0,979	0,934	1,438	1,39
60	0,979	0,934	1,456	1,408
Mean AR (AU/h),	**AR_{blank}** 0,013		**AR_{PRPP}** 0,492	

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

3. HPRT activity is calculated by the following formula:

$$\text{Activity} = \frac{AR_{PRPP} - AR_{blank}}{\varepsilon \,.\, l \,.\, [Hgb]} \times 10^6 = \frac{0.492 - 0.013}{6220 \,.\, 0.772} \times 10^6 = \mathbf{99.89\ nmol/\ mg\ of\ Hgb\ /\ h}$$

where: is the molar extinction coefficient of NADH at 340nm : $\varepsilon = 6220\ M^{-1}.cm^{-1}$

l is the path-length: l=0.772 for a 200µL round-bottom well of 96-well microplate (Corning, ref. 3797)

[Hgb], final haemoglobin concentration used in assay = 1mg/ml

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).