Application Note

A continuous spectrophotometric assay for rapid measurement of hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity in cell lysates

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Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is a central enzyme in the purine salvage pathway. The sequence of *HPRT1* gene is one of the best characterized because of its wide use as a biomarker of genotoxicity and the clinical significance of HPRT-deficiency for human health. The current methods of functional characterisation of HPRT are based on the end-point chromatographic quantification of IMP synthesized by HPRT. PRECICE® HPRT Assay Kit is a spectrophotometric, enzymatic assay that allows a continuous monitoring of IMP formation in microplate format. In the presence of recombinant IMP-dehydrogenase, IMP is oxidized to XMP with the simultaneous formation of $NADH_2$, measurable at 340nm. The specific activities of HPRT in lysates of erythrocytes, PBMC and cultured fibroblasts measured by this method are comparable to those previously determined by radiochemical procedures. PRECICE® HPRT Assay Kit allows the detection of HPRT activity as low as 6.75nmol/hour/ml with a total imprecision of 6.5%.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is a key enzyme in the purine salvage pathway. HPRT enzyme is encoded by the *HPRT1* gene, one of the best characterized genes in the human genome because of its wide use as a somatic cell genetic marker and the clinical significance of HPRT-deficiency for human health. To date, more than 300 disease-associated mutations in human *HPRT1* gene leading to partial or complete deficiencies of the HPRT enzyme have been described [1, 2]. In view of the high variability of HPRT1 gene, a rapid biochemical assay would be useful both for basic science and clinical research.

Hypoxanthine phosphoribosyltransferase 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 also catalyzes the hydrolysis of IMP and GMP, although this reverse reaction is much less favored than forward one. Importantly, human HPRT enzyme does not hydrolyze XMP.

Current procedures for characterising HPRT activity *in vitro* are based on end-point chromatographic methods, predominantly using radiochemicals. HPRT activity is then determined by measuring the rate of synthesis of IMP from radioactive precursor, hypoxanthine. This method is highly sensitive, is standardized and is currently used for the diagnostics of inherited HPRT disorders. However, such analytical methods involve several-steps handled by skilled operators, require expensive equipment and, most importantly, do not allow a large number of samples to be treated at the same time. The PRECICE® HPRT Assay Kit is a simple, rapid and cost-efficient spectrophotometric assay which, in addition, is environmentally friendly.

Since HPRT catalyzes both NMP synthesis (forward reaction) and NMP hydrolysis (reverse reaction), any of these reactions can be used for enzymatic quantification of HPRT activity. In the reverse reaction, hypoxanthine formation can be followed using xanthine oxidase but NMP hydrolysis is much less efficient than NMP synthesis, thus rendering a xanthine oxidase-based enzymatic assay not sensitive enough for convenient spectrophotometric procedure. Additionally, such an assay would not be compatible with the presence of allopurinol, a specific xanthine oxidase inhibitor which is widely prescribed to patients suffering from HPRT deficiency and that could be present in the sample. PRECICE® HPRT Assay Kit allows a real time monitoring of HPRT-catalyzed IMP synthesis by using IMP-dehydrogenase in the forward reaction which is known to be about 35-fold more efficient than the reverse one [3].

The PRECICE® HPRT Assay Kit

The PRECICE® HPRT Assay Kit is a non-radioactive, rapid assay that can be completed in 2h on a convenient standard 96-well plate and using simple absorbance measurement at 340nm. HPRT activity is measured as a rate of production of IMP. The assay is based on IMP-dehydrogenase enzyme that catalyzes the irreversible oxidation of IMP to XMP with simultaneous reduction of NAD to $NADH_2$, whose formation is directly monitored at 340nm. Figure 1 illustrates the principle of the PRECICE® Assay Kit.

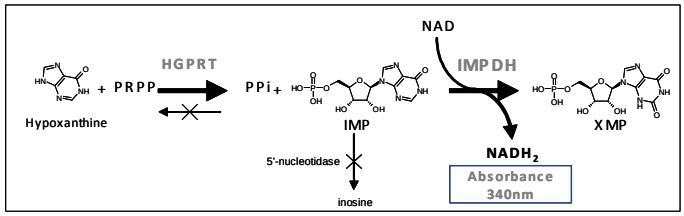

Figure 1. Enzymatic principle of PRECICE® HPRT Assay Kit

By using IMPDH enzyme for measuring HPRT activity, PRECICE® HPRT Assay Kit offers numerous advantages over end-point chromatographic assays:

(i) IMPDH enzyme is specific and selective for IMP;

(ii) IMPDH enzyme is insensitive to allopurinol, that can be present in patient hemolysate;

(iii) HPRT activity is precisely measured through a favored unidirectional reaction since the IMP produced is immediately converted by IMPDH to XMP, which is not a substrate for human HPRT, thus preventing a reverse HPRT-catalyzed reaction;

(iv) the reaction does not require any prior inactivation of 5'-nucleotidase present in cellular lysates since IMPDH enzyme is provided in large excess, thus preventing the dephosphorylation of IMP by 5'-nucleotidase enzyme

(v) HPRT activity is continuously followed by measuring the absorbance increase at 340nm and can be measured on a standard convenient 96-well plate.

Calibration with a purified Human recombinant HPRT

The calibration of the assay was done using purified human recombinant HPRT (specific activity: 3.70U/mg, concentration range: 21ng-1.5µg/ml) demonstrating IMP production rate ranging from 5.4nmol/hour/ml to 340.5nmol/hour/ml. The complete reaction mixture containing IMPDH enzyme and reagents was prepared from reaction buffer and substrates supplied in PRECICE® HPRT Assay Kit. The dilutions of human recombinant HPRT enzyme (NovoCIB, ref. E-Nov9) were made using reaction buffer without PRPP and IMPDH as the diluent. 4µL of diluted HPRT enzyme were transferred into round-bottom 96-well microplate in triplicates followed by addition of 196µL of the complete reaction mixture. After vigorous shaking for 2min, the absorbance at 340nm was monitored for 2h at 37°C using iEMS Plate Reader (Thermo Scientific). The data collected every 5 min and corrected for background absorbance (measurement done in the absence of PRPP) were used for the calculation of the rate of increase in absorbance over time (A_{340}/hour). To obtain common units (mol/hour/ml units), A_{340}/hour values were divided by the molar extinction coefficient of NADH (6220 $M^{-1}.cm^{-1}$) and the light-path length corresponding to a well of round-bottom 96-well microplate (Corning, Costar®, ref. 3797) filled with 200µL (l=0.789cm).

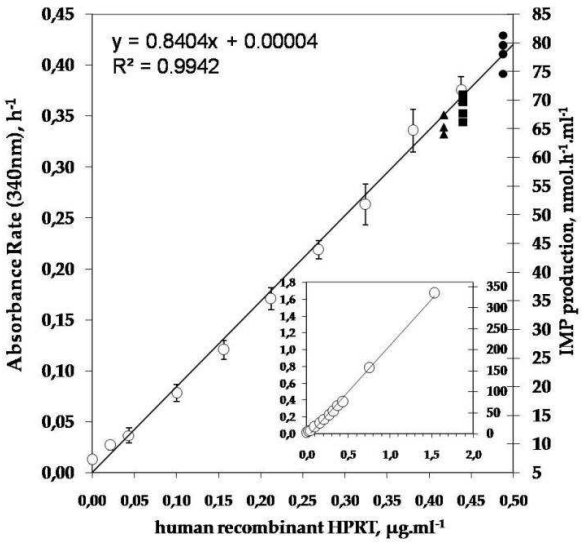

Figure 2. Calibration curve of IMPDH-based PRECICE® HPRT Assay
The rate of increase in absorbance at 340nm per hour is shown as a function of the concentration of human recombinant HPRT enzyme (NovoCIB, ref. E-Nov-9). The changes in absorbance corresponding to 3 different control hemolysates diluted in complete reaction mixture to final haemoglobin concentration 1mg/ml (n=4) are shown by filled squares, filled triangles and filled circles. The insert shows a linear correlation observed in the whole range of 21ng/ml up to 1.5µg/ml of recombinant HPRT; the units are the same.

The assay is linear up to 340nmol/hour/ml of HPRT activity with limit of detection of 6.75nmol/hour/ml (Figure 2). Within-run, between-day and total measurement imprecisions of assay run in triplicate were respectively 1.5%, 5% and 6.5% as determined at HPRT concentration 0.7µg/ml

HPRT activity in cell lysates

PRECICE® HPRT Assay was validated by measuring HPRT activity in lysates of human erythrocytes. The hemolysates were prepared by adding 4ml of water to frozen packed erythrocytes purified from 1ml of peripheral blood by Ficoll-Hypaque gradient and by sonicating for 1min on ice (Sonopuls, Bandelin, 20% cycle, 50% power). 4µL of sonicated hemolysates were added to microplate wells followed by addition of 196µL of reaction mixture to provide the initial absorbance of 0.9-1.1, optimal for monitoring the reaction. The reactions with and without PRPP (each in triplicate) were monitored in parallel at 340nm at 37°C. A_{340}/hour measured in three control hemolysates corrected for blank values varied from 0.357±0.011 to 0.412±0.015 h^{-1} at 1mg/ml final hemoglobin concentration corresponding to the range of HPRT activity of 77-89 nmol per hour per mg of hemoglobin. These values are very close to the range of HPRT activity observed in control hemolysates as determined by radiochemical procedure[4], *i.e.* 80-120nmol/h/mg

The assay was further validated by quantification of HPRT activity in lysates of peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Hypaque gradient and in lysates of cultured human fibroblasts cell line HDFα (Invitrogen). The PBMC isolated from 1.25ml of peripheral blood by Ficoll-Hypaque gradient were sonicated in 1ml of water for 1min on ice. After 1-min centrifugation at 13,000g, 100µL of PBMC lysates were added to microplate wells and completed with 100µL of a 2-fold concentrated reaction buffer (~0.15mg/ml final protein concentration). The reactions with and without PRPP (each in triplicate) were monitored in parallel at 340nm at 37°C for 4h. After the correction for the values obtained in the absence of PRPP, HPRT activities measured in PBMC of two control specimens were found to be 152.3 and 159.9 nmol/h/mg (19.7 and 21.3 nmol/h/10^6 PBMC), which is consistent with previously published data where IMP synthesis in PBMC lysates was quantified by HPLC[5]. HPRT activities as measured in sonicated lysates of cultured fibroblasts using PRECICE® HPRT Assay Kit are shown in Table 1 in comparison with published data obtained by radioactive procedure[6].

Table 1. HPRT activity in sonicates of RBC, PBMC and cultured cells

Cells	HPRT Activity		References
	PRECICE® HPRT Assay Kit	*Published data*	
RBC speciment 1 (n=4) RBC speciment 2 (n=12) RBC speciment 3 (n=4)	76.94±2.5, nmol/h/mg of Hgb 78.03±5.52,nmol/h/mg of Hgb 88.7±3.41, nmol/h/mg of Hgb	80-120 nmol/ h/mg of hemoglobin	4
PBMC specimen 1 (n=4)	159.91±3.60, nmol/h/mg of protein 19.7±0.6, nmol/h/10^6 PBMC	343±18 nmol/h/mg of protein 10.2-18.0 nmol/mg/10^6 PBMC	4 5
PBMC specimen 2 (n=4)	152.30±4.94, nmol/h/mg of protein 21.3±0.42, nmol/h/10^6 PBMC		
Human Dermal Fibroblasts (Invitrogen)	70.91±2.67, nmol/h/mg of protein	81-127 nmol/h/mg of protein	6
W20-17 cells (ATCC)	91.95±4.49, nmol/h/mg of protein		

In conclusion, a simple and sensitive non-radioactive assay for measurement of hypoxanthine-guanine phosphoribosyl-transferase activity by employing recombinant IMP-dehydrogenase enzyme was developed. By using a convenient 96-well plate, 15 samples in triplicate can be analyzed in 2h. PRECICE® HPRT Assay Kit was validated by characterising HPRT activity in lysates of RBC, PBMC or cultured cells. In addition, PRECICE® HPRT Assay Kit can be employed for high throughput screening of new anti-parasitic drugs targeting parasite HPRT since most protozoan parasites are obligate auxotroph of purines and entirely depend therefore on the enzymes of purine salvage pathways[7].

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