Continuous PRECICE® PRPP-S Assay Kit

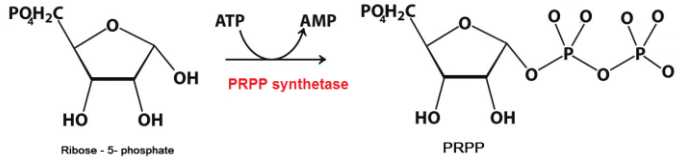
For a one-step enzymatic measurement of α-D-5-phosphoribosyl-1-pyrophosphate synthetase (PRPP-S)

I. Introduction

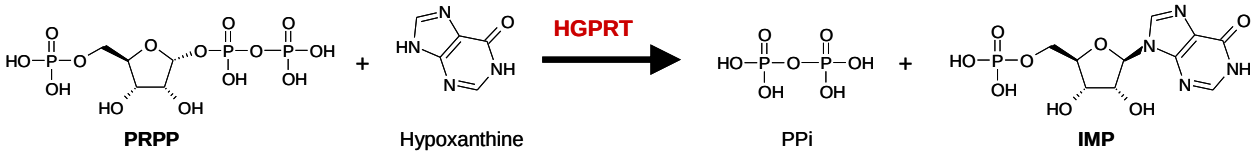
PRECICE® PRPP-S Assay Kit is designed to measure PRPP (α-D-5-phosphoribosyl-1-pyrophosphate) content in samples. This enzymatic assay is based on a coupled reaction involving Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Inosine Monophosphate Dehydrogenase (IMPDH).

The principle of the assay is based on the coupling of the following enzymatic reactions

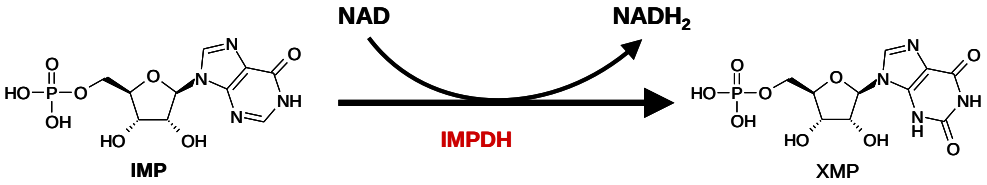
(1) In the presence of ATP and P-ribose, **PRPP-Synthetase** enzyme catalyzes the formation of PRPP



(2) In the presence of Hypoxanthine (Hx), PRPP is converted to IMP by Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to:



(3) IMP is immediately oxidized by a highly active IMPDH in the presence of NAD with simultaneous formation of $NADH_2$ directly monitored spectrophotometrically at 340 nm.



The assay is developed for measuring PRPP-S activity *in vitro* or in cell lysates.

For maximal accuracy, the assays with cell lysates are run **with and without P-ribose** 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.

II. Equipments required

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

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

III. Kit Contents for 24 analyses (12 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. The kit allows to perform 24 analysis in a time (12 samples in duplicate or 8 samples in triplicate).

A standard PRECICE® PRPP-S Assay Kit:

- one tube **"Cofactor 1"** (DTT);
- one tube **"Cofactors 2 & 3 "** (NAD and ATP);
- one tube "**IMPDH-HGPRT enzymes"**;
- one vial "**Blank"** (pre-filled with 10mL of "Reaction buffer");
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

Not provided: D-Ribose 5-phosphate (P-ribose, available at Sigma-Aldrich, ref. R7750)

***Important:* Since P-ribose solutions in water are unstable because of ubiquitous presence of phosphatases, we recommend preparing the tubes with indicated mg of P-ribose, storing as a powder at -20°C and dissolving at very last moment.**

IV. Preparation of 10ml "Reaction mixture"

1. Reconstitute the enzymes with 200µL of deionized water to the tubes with. Agitate **"IMPDH-HGPRT enzymes"** until complete dissolution of the powder.

2. Quantitatively transfer the content of 4 tubes with "**Cofactor 1", "Cofactor 2&3" and "IMPDH-HGPRT enzymes"** to "**Blank"** tube

To do so:

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

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

- 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.

3. Weight 10mg of P-Ribose (Sigma-Aldrich, ref. R7750) in a clean labeled tube (15ml)

4. Add 5ml of **"Blank"** to 10mg of P-ribose**.**

You have prepared: 5ml of **"Blank"**
5ml of **"Reaction mixture with P-Ribose"**

V. Microplate preparation

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

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

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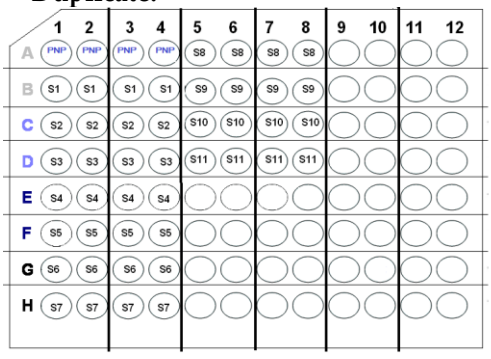

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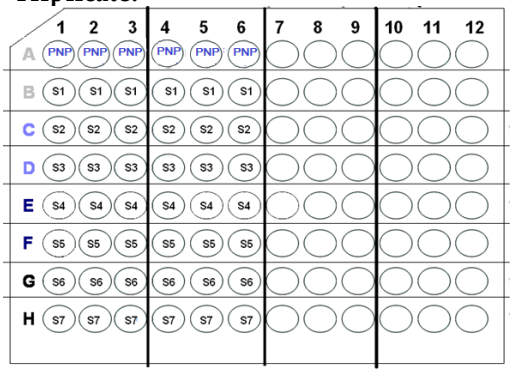

3. Add 200µL of "Blank" per well and 200µL of **"Reaction mixture"** containing **1mM P-ribose** as shown below:

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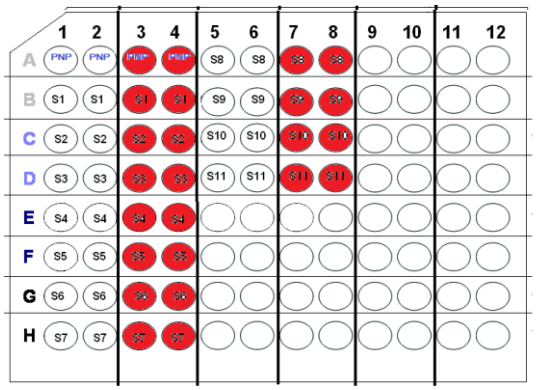

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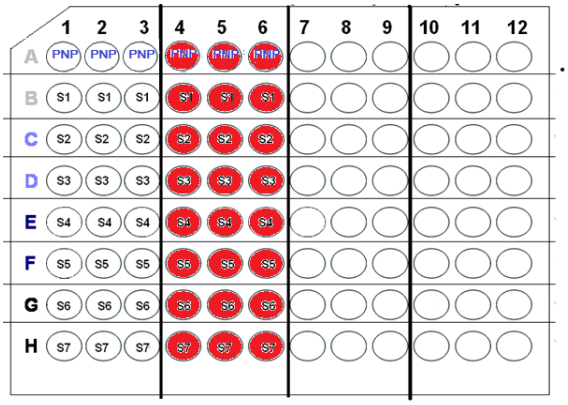

4. Program plate reader for kinetics absorbance reading (every 1 min), 37°C.

Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 1 hour with data collection every 2min. Typical results obtained with RBC lysates are shown on Table 1 / Figure 1.

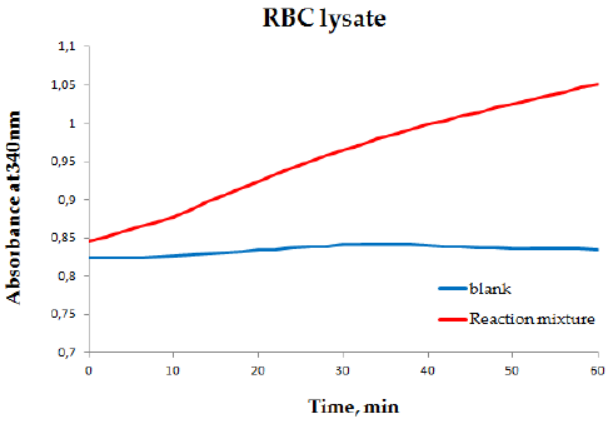

Figure 1. Kinetics of formation of PRPP catalyzed by PRPP-S in hemolysates in the absence and the presence of ribose 5-phosphate. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797)..

Time, min	Blank		Reaction mixture	
0	0,824	0,792	0,801	0,845
2	0,823	0,791	0,809	0,851
4	0,823	0,791	0,819	0,858
6	0,824	0,791	0,834	0,864
8	0,825	0,792	0,859	0,87
10	0,826	0,793	0,873	0,877
12	0,828	0,795	0,886	0,886
14	0,829	0,797	0,897	0,897
16	0,83	0,798	0,907	0,906
18	0,832	0,799	0,916	0,915
20	0,834	0,801	0,929	0,924
22	0,835	0,803	0,941	0,933
24	0,837	0,804	0,951	0,941
26	0,838	0,805	0,957	0,949
28	0,839	0,806	0,964	0,957
30	0,841	0,807	0,97	0,964
32	0,841	0,808	0,976	0,971
34	0,841	0,808	0,982	0,979
36	0,841	0,808	0,988	0,985
38	0,841	0,808	0,994	0,992
40	0,84	0,808	1	0,998
42	0,839	0,808	1,006	1,003
44	0,838	0,807	1,012	1,009
46	0,837	0,806	1,017	1,014
48	0,837	0,805	1,023	1,02
50	0,836	0,805	1,028	1,025
52	0,836	0,804	1,033	1,03
54	0,836	0,803	1,038	1,035
56	0,836	0,802	1,043	1,04
58	0,836	0,802	1,049	1,046
60	0,835	0,802	1,054	1,051
Absorbance rate per minute	0,0002415	0,0002413	0,00407	0,0035647
Absorbance rate per hour	0,0144919	0,0144798	0,2441976	0,2138831
PRPP-S activity in nmol/hour/ml			46,879937	40,693302

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V. Calculation of PRPP-S activity in hemolysates

1. Calculate the absorbance rate per hour for reaction buffers with Ribose 5-phosphate (ARP5R) and without (ARblank).
2. Calculate Mean ARP5r and Mean ARblank
3. Measure the concentration of hemoglobin [Hgb] in hemolysates using Drabkin's reagent and calculate final [Hgb] concentration used in assay.
4. PRPP-S activity is calculated by the following formula:

$$\text{Activity} = \frac{\text{Mean AR}_{P5R} - \text{Mean AR}_{blank}}{4.9 \times [\text{Hgb}]} \times 10^3 = \frac{(0.229 - 0.014)}{4.9 \times 0.62} \times 10^3 = 71 \text{ nmol/ hour / mg of Hgb}$$

Where: Mean AR_{P5R} = 0.229

Mean AR_{blank} = 0.014

[Hgb], final haemoglobin concentration used in assay = 0.62 mg/ml

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