Continuous PRECICE® PRPP-S Assay Kit

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

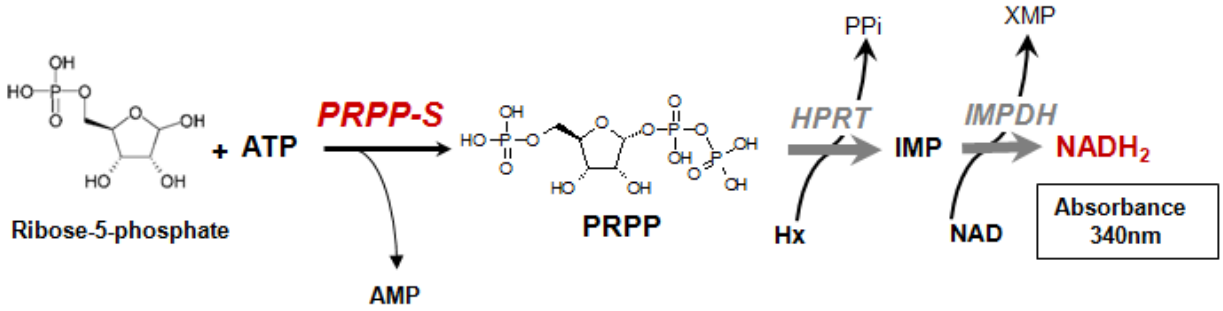
I. Introduction

PRECICE® PRPP-S Assay Kit provides the first **non radioactive** and one-step protocol for measurement of PRPP-S activity in cellular lysates in a convenient 96-well plate format. This enzymatic assay is based on a coupled reaction involving Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Inosine Monophosphate Dehydrogenase (IMPDH).

Phosphoribosylpyrophosphate synthetase (PRS; EC 2.7.6.1), an essential enzyme for the purine salvage pathway, is encoded by PRPS1 gene. Several mutations in this gene associated with genetic disorders have been described leading to **PRS superactivity**. This condition is inherited in an X-linked pattern. PRPS1 gene overactivity increases the production of normal PRPP synthetase 1 enzyme, which increases the availability of PRPP. Excessive amounts of purines are generated leading to an accumulation of uric acid, a waste product of purine breakdown, in the body. A buildup of uric acid can cause gout, which is a form of arthritis resulting from uric acid crystals in the joints. Affected individuals may also develop kidney or bladder stones formed from uric acid crystals.

Increase of the availability of PRPP can be due to PRPP-S superactivity or HPRT deficiency

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)

(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 Ribose-5-Phosphate** in parallel. The absorbance rate observed in the absence of Ribose-5-Phosphate 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 (8 samples in triplicate):

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 performing 24 analyses in a time (8 samples in triplicate or 12 samples in duplicate).

A standard PRECICE® PRPP-S Assay Kit contains:

- one tube **"Cofactor 1"**;
- one tube **"Cofactors 2 & 3"**;
- one tube "**Enzymatic mix**";
- one tube "**10X buffer**" (pre-filled with 1 ml of 10X buffer)**;**
- one 15mL tube "**Blank"** (orange cap);
- one 15mL tube **"Reaction mixture with P-Ribose"** (blue cap) (pre-filled with 25µmol of P-Ribose);
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

IV. Preparation of 10ml "Reaction mixture"

1. Transfer the content of the tube "**10X buffer**" into the 15mL tube "**Blank"** (orange cap) and add 9mL of deionized water. 10mL of 1X buffer is obtained.

2. Quantitatively transfer the content of 3 tubes with "**Cofactor 1", "Cofactors 2&3", and "Enzymatic mix"** 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 is dissolved.

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

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

3. Transfer 5ml of complete Reaction mixture 1x containing enzymes and cofactors to blue cap 15ml tube pre-filled with P-ribose (powder)**.**

You have prepared: 5ml of **"Blank"**
5ml of **"Reaction mixture with 5mM 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-S12) per well as shown next page:

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 PRPP-S 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).

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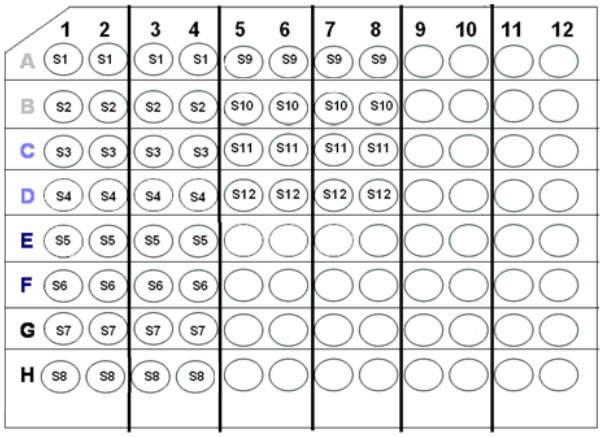

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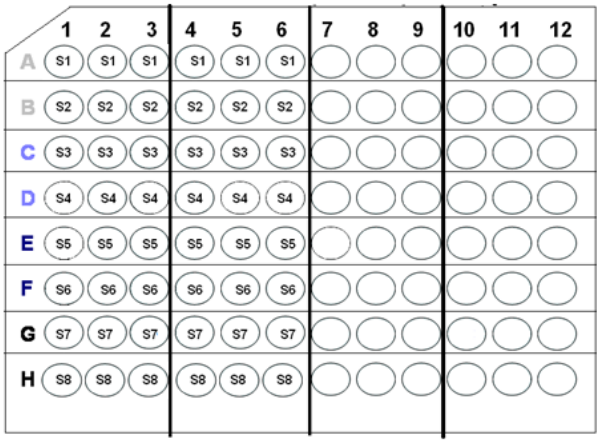

3. Add 200µL of "Blank" per well and 200µL of **"Reaction mixture"** containing **5mM Ribose 5-phosphate** as shown below:

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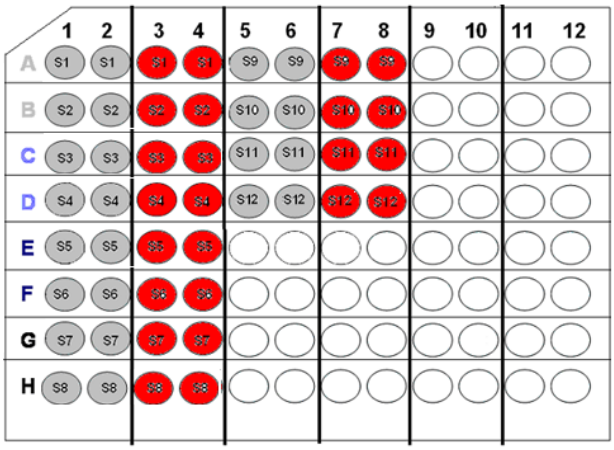

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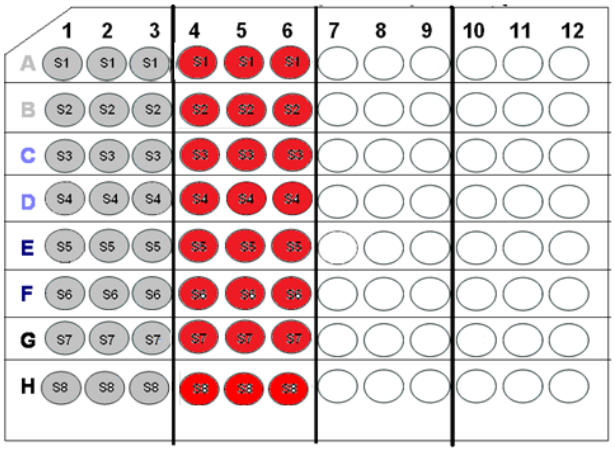

4. Program plate reader for kinetics absorbance reading (every 2min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 1 hour with data collection every 2min. Typical results obtained with RBC lysates are shown on Table 1 / Figure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

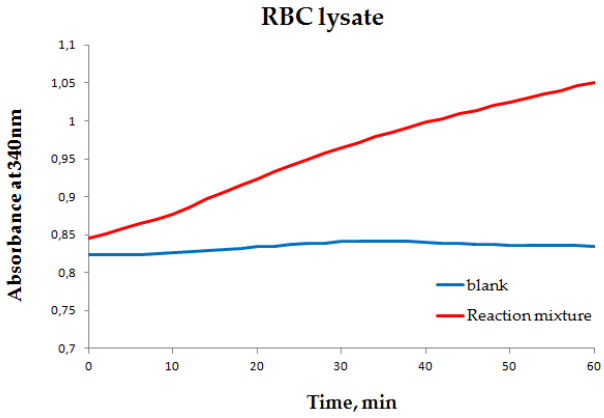

Figure1. Kinetics of formation of PRPP catalyzed by PRPP-S in hemolysates in the absence and the presence of ribose 5-phosphate. **After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, 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

VI. Calculation of PRPP-S activity in hemolysates

1. Calculate the absorbance rate per hour for reaction buffers with Ribose 5-phosphate (AR_{P5R}) and without (AR_{blank}).

2. Calculate Mean AR_{P5r} and Mean AR_{blank}

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).