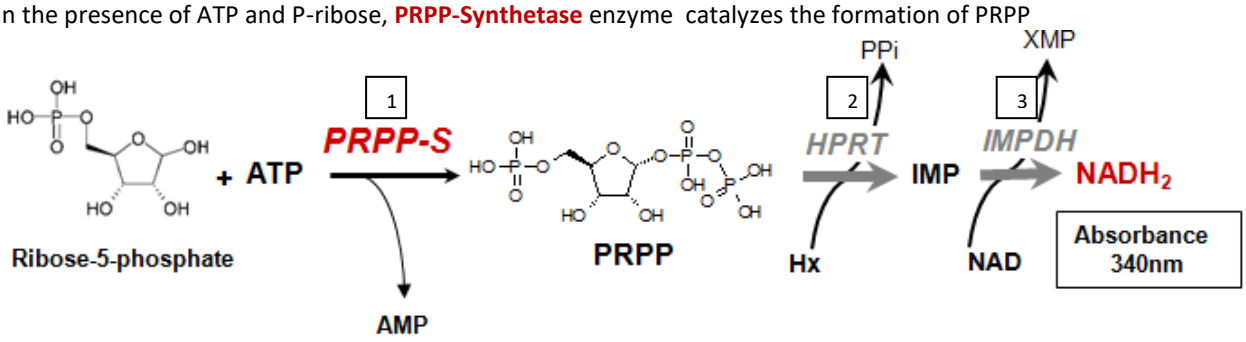
PRECICE® PRPP-S Assay Kit:
User manual - Ref: # K0709-04-2

I. Introduction

PRECICE® PRPP-S Assay Kit is designed for continuous monitoring of PRPP synthesis. The assay is based on coupling of two recombinant enzymes : Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Inosine Monophosphate Dehydrogenase (IMPDH).

(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 P-ribose** in parallel. The absorbance rate observed in the absence of P-ribose 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 10mL of reaction mixture):

Once dissolved, the reagents can be stored at -20°C for three months.

A standard PRECICE® PRPP-S Assay Kit:

- **Cysteine**;
- **NAD;**
- **ATP** ;
- **P-ribose;**
- **HPRT and IMPDH enzymes, lyophilized**
- **Reaction buffer** (glass vial, 10mL) ;
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

The kit is shipped at room temperature since dry reagents and lyophilized enzymes are stable at room temperature (up to 2 weeks). However, for long time storage the kit should be frozen upon arrival and stored at -20°C.

IV. Preparation of 1ml "Reaction mixture"

IMPORTANT: Use only autoclaved Milli-Q water to inactivate ubiquitous phosphatases and to avoid dephosphorylation of P-ribose and PRPP present in reaction mixture

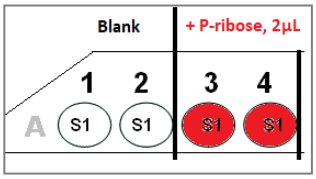
1. Shortly spin the tubes before opening to recover the powder at the bottom;
2. Thaw "**Reaction buffer"** (do not heat); equilibrate at room temperature;
3. Add 200µL of deionized water to the tube with "**HPRT and IMPDH enzymes",** agitate **(**do not vortex to avoid foam) and spin shortly;
4. Add 100µL of deionized water to each of four tubes (**Cysteine**, **NAD**, **ATP** and **P-ribose)**. Vortex until complete dissolution, spin shortly;
5. Put 0.85mL of reaction buffer in a clean 1.5mL tube, add
   - 9µL of **"Cysteine",**
   - 9µL of **"NAD"**
   - 9µL of **"ATP"** solutions

   Do not add **P-ribose** solution
6. Close and agitate by inverting;
7. Add 18µL of **"HPRT-IMPDH enzyme"** solution; close and agitate by inverting, spin shortly;

Composition of reaction mixture: 100mM Tris-HCl, 100mM KCl, 12mM MgCl2, 40mM KH2PO4, BSA 1mg/ml, 1mM hypoxanthine, 1mM NAD, 4mM cysteine, 2.5 mM ATP, IMPDH-HGPRT 50mU/ml each, pH8.5, start by P-ribose (3.5mM)

V. Reaction monitoring

1. Program plate reader for kinetics absorbance reading (every 1 min), 37°C.
2. Add desired amount of PRPP-S solution (1-10µL) per well to four wells, followed by addition of 200µL of **"Reaction mixture"**
3. Insert the plate into the reader pre-heated at 37°C, agitate for 1min and incubate for 15 min;
4. To start the reaction, add 2µL of P-ribose solution to two wells (two other will be used as Blank), agitate and monitor the reaction at 340nm at 37°C for 1 hour with data collection every min.



VI. Calculating PRPP-S activity (U/ml)

Typical results obtained with RBC lysates are shown on Table 1 / Figure 1.

1. Calculate the absorbance rate per min for reaction buffers with Ribose 5-phosphate (AR) and without (AR_{blank}) using "Slope" function of Excel.
2. Calculate mean values for $AR_{P\text{-}Rib}$ and AR_{blank}
3. Calculate PRPP-S activity (U/ml) using following formula:

$$\text{Activity (U/ml)} = \frac{(AR - ARblank) * dilution\ factor}{4{,}9}$$

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

1 Unit (U) is defined as 1 µmol per min

Dilution factor = well volume (200µL) / added volume (1-10µL)

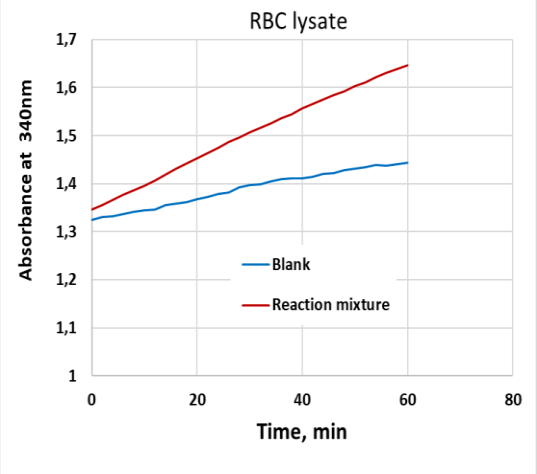


Figure 1. Kinetics of formation of PRPP catalyzed by PRPP-S in hemolysates in the absence and the presence of ribose 5-phosphate. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®,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

Table 1.

Time, min	Blank	Blank	**P-ribose**	**P-ribose**
0	1,325	1,29	1,383	1,346
2	1,331	1,291	1,391	1,356
4	1,333	1,297	1,398	1,366
6	1,337	1,3	1,41	1,377
8	1,341	1,296	1,421	1,387
10	1,345	1,299	1,433	1,396
12	1,347	1,304	1,444	1,407
14	1,355	1,307	1,458	1,419
16	1,358	1,309	1,47	1,431
18	1,361	1,312	1,482	1,442
20	1,368	1,316	1,492	1,453
22	1,373	1,322	1,502	1,464
24	1,379	1,325	1,513	1,475
26	1,382	1,332	1,523	1,487
28	1,393	1,336	1,535	1,496
30	1,398	1,339	1,547	1,507
32	1,399	1,338	1,557	1,517
34	1,405	1,334	1,565	1,526
36	1,409	1,342	1,572	1,536
38	1,411	1,342	1,579	1,545
40	1,412	1,347	1,589	1,556
42	1,415	1,348	1,603	1,566
44	1,42	1,35	1,61	1,576
46	1,422	1,354	1,616	1,584
48	1,429	1,355	1,627	1,593
50	1,431	1,357	1,637	1,603
52	1,434	1,362	1,649	1,611
54	1,439	1,361	1,658	1,621
56	1,437	1,368	1,665	1,631
58	1,441	1,367	1,671	1,639
60	1,443	1,37	1,68	1,647

	Blank		**P-ribose**	
Absorbance Rate per min (AR, AU/min)	0,0016	0,0012	**0,0047**	**0,0047**
AR, mean, AU/min	0,0014		**0,0047**	
AR after blank subtraction, AU/min			**0,0033**	
	Dilution factor (4µL of RBC lysate per 200-µL well)		1mM NADH absorbance, (AU)	
	50		4,90	
PRPP-S activity in RBC lysate, U/ml (µmol/min/ml)			**0,034**	

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