

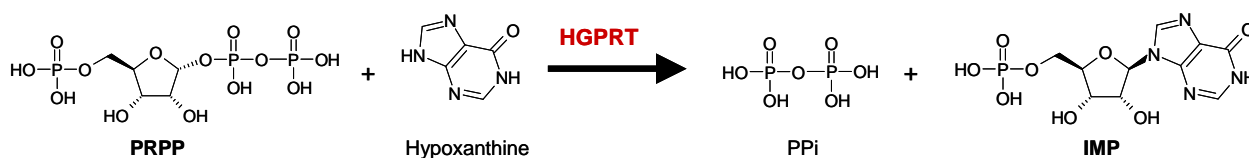
PRECICE® PRPP Assay Kit
For a one-step enzymatic measurement of α -D-5-phosphoribosyl-1-pyrophosphate (PRPP)

I. Introduction

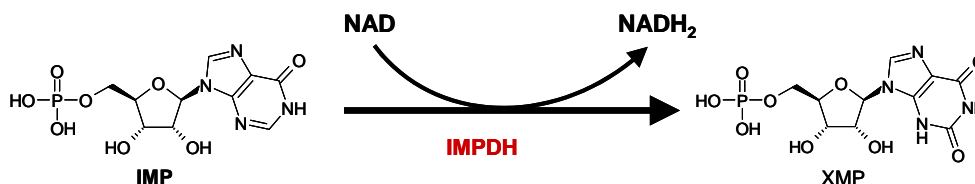
PRECICE® PRPP Assay Kit is designed to measure PRPP (α -D-5-phosphoribosyl-1-pyrophosphate) content in samples. This enzymatic assay is based on a coupled reaction involving Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Inosine Monophosphate Dehydrogenase (IMPDH). PRPP is highly unstable, which renders the preparation of accurately weighted standards difficult. **NOVO**CIB's PRECICE® PRPP Assay Kit offers the advantage of measuring PRPP concentration in the sample by absorbance through a stoichiometric NADH₂ formation, thus allowing a direct and absolute measurement of PRPP content with no need of any calibration curve.

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

- (1) In the presence of Hypoxanthine, the 5-phosphoribosyl group of PRPP is first transferred by Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to form Inosine Monophosphate (IMP) and release a pyrophosphate:



- (2) IMP is immediately oxidized to Xanthosine Monophosphate (XMP) by a highly active IMPDH in the presence of NAD.



The NADH₂ formed is equivalent to the amount of PRPP present in the assay. NADH₂ formation is directly monitored spectrophotometrically at 340 nm.

II. Kit Content

A standard 100-assay PRECICE® PRPP Assay Kit contains a set of 6 tubes consisting in:

- one tube of lyophilized IMPDH (≥ 500mU).....Tube "IMPDH"
- one tube of lyophilized HGPRT (≥ 500mU).....Tube "HGPRT"
- one tube of hypoxanthine solution at 20mM (≥ 1mL).....Tube "Hx"
- one tube of NAD (powder).....Tube "NAD"
- two tubes containing 10x concentrated Buffer (≥ 1mL)
(Tris-HCl 1M pH 8, KCl 1M, MgCl₂ 120 mM).....Tube "TKM Buff x10"

A set of these 6 tubes is sufficient to prepare 20 ml of reaction mix, i.e. 100 assays under standard conditions hereafter described (one 96-well plate with 200μL of reaction mix per well).

Higher sized-PRPP Assay Kits are provided with the adequate number of sets of tubes.

DTT and deionized water are not provided.

IMPORTANT: The following instructions are given to measure the concentration of PRPP, in a range allowing this measurement by spectrophotometry, for R&D use only. NovoCIB does not guarantee the use of its PRECICE® PRPP Assay Kit or of one or several of its component, in other conditions and/or for other purpose than those described in this user manual.

III. Method and Conditions

PRPP concentration of is measured by a spectrophotometric determination of absorbance at 340nm, 37°C, pH=8

IV. Preparation of Stock Solutions of reagents and IMPDH and HGPRT enzymes

Using stock solutions of hypoxanthine, NAD and IMPDH and HGPRT enzymes is particularly convenient to run assays as and when needed. Hypoxanthine is already provided in a stock solution at 20mM. However, NAD and IMPDH and HGPRT enzymes Stock Solutions must be prepared according to the following instructions.

IV.1. Preparation of NAD Stock Solution

- For 100 assays of 200µL (20mL total), prepare 200µL of 0.1M NAD by dissolving NAD in 200µL of deionized water.
- Stock Solution of NAD (x100) is then ready for use. It is stable for several weeks when stored at -20°C.

IV.2. Reconstitution of IMPDH enzyme

- Refer to the label of the tube containing lyophilized IMPDH.
 - Add the indicated volume of deionized water* to reconstitute the lyophilized IMPDH.
 - Before use, prepare a ~1U/mL IMPDH solution by diluting (if needed) the reconstituted IMPDH with TKM Buffer x1
- * If storage after reconstitution is needed, add 50%-glycerol/deionized water solution instead of deionized water. The reconstituted enzyme in 50%-glycerol can then be stored for several weeks at -20°C.

IV.3. Reconstitution of HGPRT enzyme

- Refer to the label of the tube containing lyophilized HGPRT.
 - Add the indicated volume of deionized water* to reconstitute the lyophilized HGPRT.
 - Before use, prepare a ~1U/mL HGPRT solution by diluting (if needed) the reconstituted HGPRT with TKM Buffer x1
- * If storage after reconstitution is needed, add 50%-glycerol/deionized water solution instead of deionized water. The reconstituted enzyme in 50%-glycerol can then be stored for several weeks at -20°C.

NAD and Hypoxanthine Stock Solutions are stable for few weeks when stored at -20°C. HPRT and IMPDH solutions are stable at -20°C for few weeks only when reconstituted in 50% glycerol solution.

V. Preparation of the reaction mix (standard procedure)

The volume of reaction mixture (V, mL) to prepare will depend on the number of assays to run. Here below are the procedures for **X assays (V = X . 0.2mL)**, **12 assays (V = 2.4mL)** and **100 assays (V = 20mL)**

	Total Volume required	X assays V = X . 0.2 mL	12 assays V = 2.4 mL	100 assays V = 20 mL
<u>V.1. Prepare into suitable containers:</u>				
<ul style="list-style-type: none"> • deionized water: >V mL • freshly prepared 1M Dithiothreitol (DTT) solution in deionized water 		<ul style="list-style-type: none"> • > 0.2 X mL • > 40 µL 	<ul style="list-style-type: none"> • > 2.4 mL • > 40 µL 	<ul style="list-style-type: none"> • > 20 mL • > 40 µL
<u>V.2. Prepare the reaction buffer</u>				
<ul style="list-style-type: none"> • In a sterile tube, pipet 1/10 V mL of 10x concentrated buffer (Tube "TKM Buff x10") • Add 17/20 V mL of deionized water • Add 2V µl of freshly prepared 1M DTT solution (<i>i.e. dilute 500x</i>) 		<ul style="list-style-type: none"> • 20 X µL • 0.17 X mL • 0.4 X µL 	<ul style="list-style-type: none"> • 240 µL • 2.04 mL • 4.8 µL 	<ul style="list-style-type: none"> • 2 mL • 17 mL • 40 µL
<u>V.3. Add the reagents and IMPDH enzyme from Stock Solutions (previously prepared as described in section IV)</u>				
<ul style="list-style-type: none"> • NAD Stock Solution at 100mM • IMPDH enzyme solution at ~1mU/µL • HGPRT enzyme solution at ~1mU/µL 	<p>To dilute 100x</p> <p>To dilute 40x</p> <p>To dilute 40x</p>	<ul style="list-style-type: none"> • 2 X µL • 5 X µL • 5 X µL 	<ul style="list-style-type: none"> • 24 µL • 60 µL • 60 µL 	<ul style="list-style-type: none"> • 200 µL • 500 µL • 500 µL
<ul style="list-style-type: none"> • Mix gently by inversion of the tube or by pipetting 				

Reaction mix is ready (Buffer Tris-HCl 100mM pH 8, KCl 0.1 M, MgCl₂ 12 mM, NAD 1mM, IMPDH ~25mU/ml, HGPRT ~25mU/ml). Reaction is started by adding Hypoxanthine (see the following section, VI).

VI. PRPP measurement

Warning: Efficiency of PRECICE[®] PRPP Assay Kit for PRPP measurement depends upon PRPP final concentration in the assay, which should be in a range of 20 to 400µM final concentration (i.e. in the assay) to be confidently measured in a 200µL-well.

This assay was validated using standard solutions of PRPP. Measurement of PRPP concentration in complex sample, for instance containing various reagents and/or enzymes, may disturb HGPRT-IMPDH coupled reaction and give erroneous PRPP measurement.

Following instruction are given for a relatively highly PRPP-concentrated sample, diluted 10-100 fold in the reaction mix, leading final concentration of PRPP ranging from 20µM to 400µM.

VI.1. Pre-incubation (10')

- Program the plate-reader in a Kinetics mode, 340 nm, 37°C
- Pipette between 2-20µl of sample in the wells.
- Add reaction mix to complete at 200µL.

Important: it is strongly advised to run duplicate assays and to run in parallel a negative control with sample, reaction mix but without starting the reaction (no Hx added).

- Pre-incubate in the plate-reader at 37°C and measure A_{340nm} every 1 or 2 minutes for 10mn. Absorbance should stabilize during the pre-incubation phase. Record this first set of data.

VI.2. Start the reaction and incubate for 20 to 40mn (until a plateau is reached)

- Eject the plate from the plate-reader
- Start the reaction by adding 10µL of hypoxanthine solution at 20mM (vial "Hx") in the assay. Keep negative control without Hx!
- Replace the plate in the plate-reader and measure A_{340nm} every 1 or 2 minutes for 20-40mn, eventually more until a plateau is reached.

VI.3. Data analysis and calculation

For each well, including for negative control assays, read the absorbance $A(t_0)$ at the end of the pre-incubation phase and the absorbance $A(t_f)$ when a plateau is reached, at the end of the incubation phase.

PRPP concentration of the sample is then calculated through the following equation:

$$[\text{PRPP}] \text{ in the sample (in } \mu\text{M)} = \frac{(A^a(t_f) - A^a(t_0)) - (A^c(t_f) - A^c(t_0))}{\epsilon \cdot l \cdot V_{\text{sample}}} \times 2.10^8$$

where: ϵ is the molar extinction coefficient of NADH at 340nm : $\epsilon = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$
 A^a and A^c are the Absorbance of the assay and the absorbance of the negative control, respectively
 l is the path-length in a 200µL-well
 V_{sample} is the volume of sample used in the assay, expressed in µL (between 2 and 20µl)

Example of PRPP concentration measurement using a range of PRPP standard

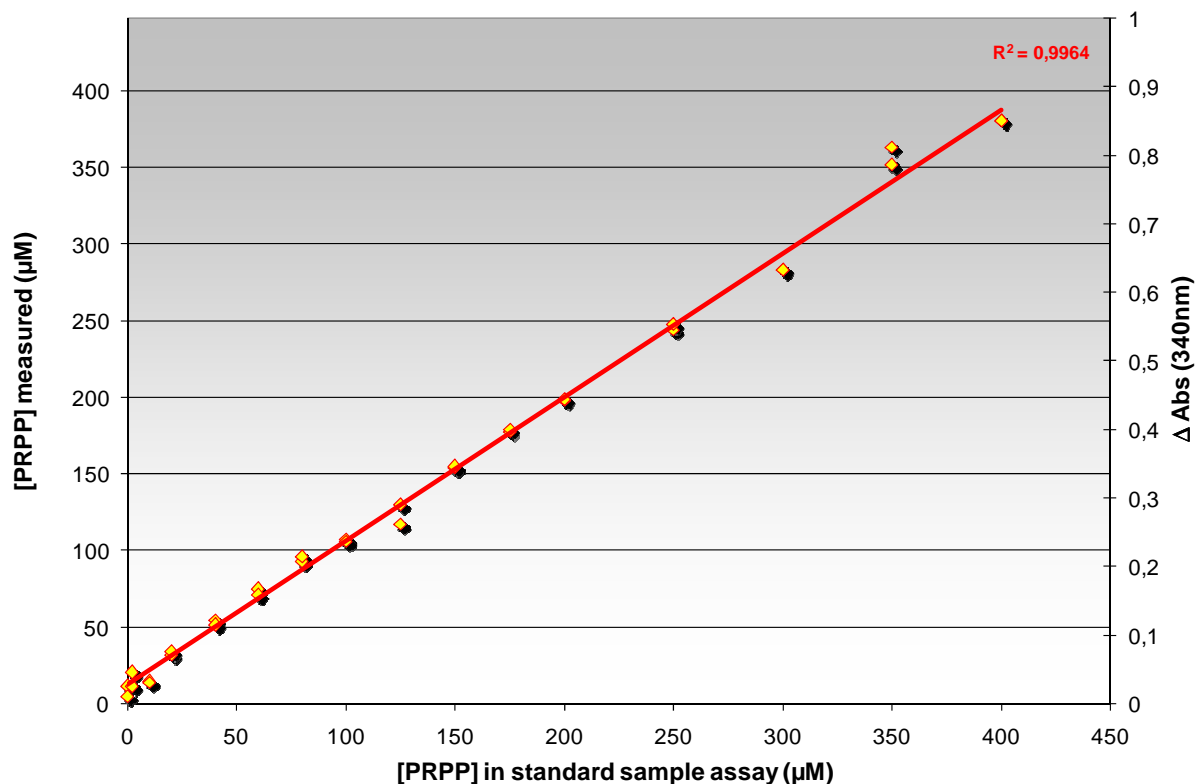


Figure 1: Correlation between PRPP concentrations of the standards and PRPP concentrations measured with NOVO CIB's PRECICE[®] PRPP Assay Kit.

Assays were done in duplicate using a range of freshly prepared PRPP (Sigma-Aldrich, P8296). The concentration of the standards indicated on the graphic takes into account the 75% purity indicated by the the supplier. Due to PRPP instability, standards may be difficult to prepare. This correlation curve shows that a direct measurement of PRPP content can be done without any standard but directly through the absorbance of the NADH₂ formed in the assay after 35 minutes of reaction.