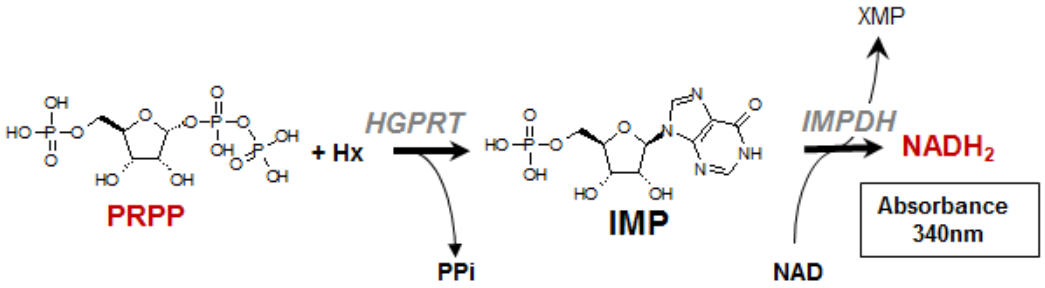
PRECICE® PRPP Assay Kit

For enzymatic measurement of α-D-5-phosphoribosyl-1-pyrophosphate (PRPP)

PRECICE® PRPP Assay Kit is a first non-radioactive assay for *in vitro* measurement of PRPP (α-D-5-phosphoribosyl-1-pyrophosphate) content in cells extracts. This enzymatic assay is based on a coupled reaction involving Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Inosine Monophosphate Dehydrogenase (IMPDH).

I. Principle

In the presence of hypoxanthine, HGPRT enzyme converts PRPP to IMP which is further oxidized to XMP by downstream IMPDH enzyme leading to simultaneous $NADH_2$ formation. The amount of $NADH_2$ formed is mesured spectrophotometrically at 340 nm and is equivalent to the amount of PRPP in the assay.



Short description of procedure:

1. PRPP is extracted from cells with organic solvent.
2. Clarified and filtered extracts are dried under reduced pressure.
3. Dried extracts are resuspended in 250µL of **Reaction mixture** (containing HGPRT-IMPDH enzymes) or with 250µL of **BLANK** (containing IMPDH enzyme only**)**.
4. The absorbance of samples is measured at 340nm. The absorbance observed in the absence of HGPRT is used as blank and is subtracted from the absorbance measured in its presence.

Warnings:

1. **Method sensitivity.** PRECICE® PRPP Assay Kit allows PRPP quantification within the range of 8 to 400µM. Since, according to published data, PRPP concentration in mammalian cells varies from 0.1 to 1µM, the extracts should be concentrated 10-100 folds to allow spectrophotometric quantification. If the absorbance is measured using plate reader, the volume of 96-well plate is 200µL. The volume of cells to be extracted should be at least 2mL. Theses volumes can be reduced by reading the results with spectrophotometer and using small volume spectrophotometer cuvette (50µL).
2. **PRPP unstability.** PRPP is highly unstable cellular metabolite susceptible to degradation by phosphatases and nucleotidases present in all cell types and organisms from prokaryotes to eukaryotes. To avoid PRPP degradation by phosphatases and nucleotidases: all aqueous solutions (deionized water, PBS) should be autoclaved for 30 min; cellular enzymes should be totally precipitated during extraction step.
3. **Control PRPP degradation** using positive control where PRPP (10µL of freshly prepared 10mM solution) is added to filtered cellular extracts or to cells suspension.

NB: The difficulties of measurement of PRPP in cells due to its low intracellular concentration and high unstability can be circumvent by measuring the activity of PRPP-synthetase done in one step.

II. Equipments required

1) Plate agitator
2) Plate reader fitted with a filter 340nm or spectrophotometer.
3) Polypropylene tubes (ex. Corning, ref. 430829)
4) PTFE syringe filters (0.2µm)

III. Kit Contents for 24 analyses (8 samples in triplicate):

Once dissolved, the reagents provided in the kit are not stable and should be used immediately.

A standard PRECICE® PRPP Assay Kit:

- one tube **"Cofactor 1"** (DTT);
- one tube **"Cofactor 2"** (NAD);
- one tube "**IMPDH enzyme**" (1U);
- one tube "**Reaction Mixture**" (HGPRT, 1U);
- one tube "**BLANK";**

(0.5M Tris-HCl; 0.11MKCl, 13mMMgCL2 autoclavé, puis ajouté BSA FA-free chauffé 70°C 1h BSA1mg/ml, contains 0.5mM hypoxanthine et 50mM KF);

- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

Not provided:

Autoclaved deionized water

Positive control: PRPP (α–D-5-phosphoribosyl-1-pyrophosphate, available at Sigma-Aldrich, ref. P8296)

Acetone

IV. Recommendations for PRPP extraction / concentration:

Several protocols of PRPP extraction have been described:

- *Chemical extraction where proteins are precipitated with perchloric acid (PCA) or trichloroacetic acid (TCA).*
- *Extraction by boiling where cells or tissue is heated for 85°C.*

Since PRPP is stable neither at acidic pH nor at high temperature, NovoCIB has developed an alternative method of PRPP extract based on protein precipitation with organic solvent. The protocol of acetone extraction is described below. ***This protocol should be validated to every cell type and optimized for cell number to be extracted.*** *For additional information contact us at contact@novocib.com*

Important:

Use only polypropylene tubes (ex. - Corning, ref. 430829) resistant to acetone. Never use polystyrene tubes.

For 2ml of cell pellet (to be prepared in duplicate):

1. *Chill 50 mL of acetone at-70°C.*

2. *Prepare pellet of cells (2-3mL) extensively washed with autoclaved PBS solution;*
   Recover cells by centrifugation. Carefully remove supernatant, wash cell pellet with autoclaved PBS.
   Repeat 3 times to remove all nucleotidases and phophatases present in medium or serum. Carefully remove all PBS.

3. *Chill cell pellet on ice for 5 min.*

4. *Transfer 50mL of cold acetone to cell pellet, put immediately at -70°C.*

5. *Incubate for 3h at -70°C (or overnight at -20°C) to precipitate all proteins.*

6. *Centrifuge the extracts at maximal speed for 30 min at 4°C, carefully recover supernatant (acetone extract) that should be totally clear. While recovering supernatant, avoid to touch and to resuspend protein pellet.*

7. *Filter acetone extract using PTFE syringe filters (0.2µm) to remove all proteins not precipitated by centrifugation.* ***The presence of remaining proteins in acetone extract would lead to PRPP degradation during drying of the extracts.***

The efficacy of protein precipitation by acetone depends on temperature, protein concentration and can vary for different cell types. Protein precipitation can be improved by using the solvents other than acetone or by adding carrier protein.

V. Preparation of enzymatic reaction mixtures

1. Add 100µL of autoclaved deionized water to the tube with **"IMPDH enzyme**.

2. Vigorously agitate the vial "**BLANK"** before opening.

3. Quantitatively transfer the content of 3 tubes with "**Cofactor 1", "Cofactor 2**" and **"IMPDH enzyme"** to a tube "**BLANK"**

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.

6. Transfer 5ml of thus prepared **"BLANK"** to a vial **"Reaction mixture"** pre-filled with lyophilized HGPRT enzyme.

You have prepared: 5ml of "**BLANK"**
5ml of **"Reaction mixture"**

VI. Drying extracts and measuring absorbance

1. Split filtered acetone extract into 2 glass vials.
2. Dry the extracts under reduced pressure. The faster drying provides better results.
   Solubilize one half of dried acetone extract by addition of 250µL of "**BLANK"** (containing IMPDH enzyme**)** and another half with 250µL of **Reaction mixture** (containing HGPRT-IMPDH enzymes). Incubate for 30 min at room temperature.
3. Carefully recover solubilized extracts, centrifuge at 13 000g for 10min. The extracts should be totally clear. If the extracts are not clear, add 50µL of ethanol and centrifuge again.
4. Program plate reader for absorbance reading (absorbance at 340nm), insert the plate into the reader and read absorbance data.

Spectrophotometer and 50µL cuvette:

1. Resuspend dried extract in 100µL of "Blank" or "Reaction buffer". Incubate for 30 min at room temperature.
2. Carefully recover the extracts, centrifuge at 13 000g for 10min. The extracts should be totally clear. If the extracts are not clear, add 50µL of ethanol and centrifuge again.
3. Blank the spectrophotometer using cuvette filled with water. Measure the absorbance of samples resuspended in "Blank" and "Reaction buffer" at 340nm.

VII. Positive controls:

1. Prepare fresh solution of 10mM PRPP in 10mM NaF solution in water.
2. Add 10µL of 10mM PRPP to 40mL filtered extracts. Split the extract into 2 glass vials and proceed with drying.

   This positive control allows checking the absence of phosphatases/nucleotidases in cellular extracts and PRPP degradation during drying. The expected optical density (340nm) of positive control after "BLANK" subtraction should be 1.225.

   To exclude PRPP degradation during extraction, you can add 10µL of 10mM PRPP to cells suspension before or immediately after acetone addition.

VIII. Calculating PRPP concentration

Plate reader:

$$[\text{PRPP}] \text{ in the sample (in } \mu\text{M}) = \frac{\text{Absorbance}_{\text{Reaction}} - \text{Absorbance}_{\text{Blank}}}{0.0049 *}$$

Where: 0.0049 is extinction coefficient of 1µM NADH at 340nm corrected for l (path-length of round-bottom 200µL-well 96-well plate Corning, Costar®, ref. 3797) as measured with iEMS Labsystems plate reader (Thermo).

Spectrophotometer:

$$[\text{PRPP}] \text{ in the sample (in } \mu\text{M}) = \frac{\text{Absorbance}_{\text{Reaction}} - \text{Absorbance}_{\text{Blank}}}{0.0062 *}$$

Where: 0.0062 is extinction coefficient of 1µM NADH at 340nm in 1cm cuvette.