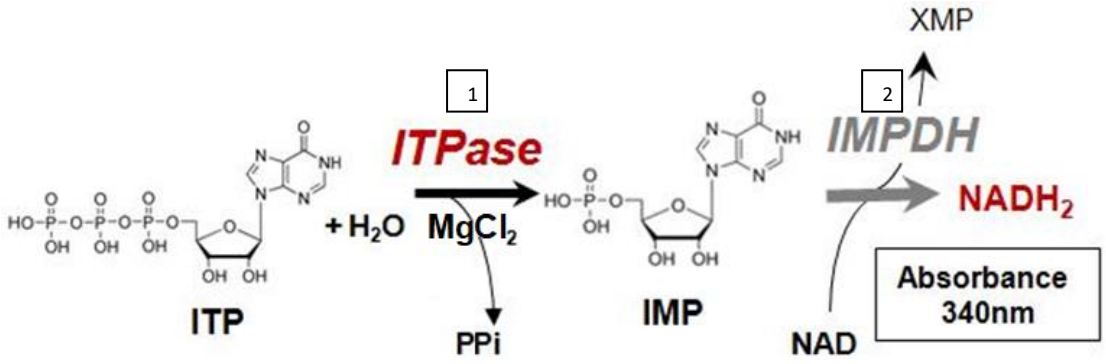
PRECICE® ITP pyrophosphatase (ITPase) Assay Kit: User manual - Ref: # K0709-06-2

I. Introduction

PRECICE® ITPase Assay Kit is designed for continuous monitoring of ITP-pyrophosphatase activity. The assay is based on a reaction involving Inosine Monophosphate Dehydrogenase (IMPDH).



(1) In the presence of Mg^{2+} **ITPase** enzyme hydrolyzes ITP to IMP and PP_i;

(2) Released 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 ITPase activity *in vitro* or in cell lysates. *For maximal accuracy,* the assays are run **with and without ITP** in parallel. The absorbance rate observed in the absence of ITP 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 ITPase enzyme, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® ITPase 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):

A standard PRECICE® ITPase Assay Kit:

- **Cysteine** (powder);
- **NAD** (powder)
- **ITP** (powder)
- **IMP** (powder, positive control for IMPDH)
- **IMPDH enzymes, lyophilized**
- **Reaction buffer** (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.

Once dissolved, the reagents should be stored at -20°C and used within three months.

IV. Preparation of "Reaction mixture" for one sample in duplicate (4 wells of 200µL each)

IMPORTANT: Use only autoclaved Milli-Q water to inactivate ubiquitous phosphatases

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 "**IMPDH enzyme",** agitate (do not vortex to avoid foam) and spin shortly;
4. Add 100µL of deionized water to each of four tubes (**Cysteine**, **NAD, ITP and IMP)**. Vortex until complete dissolution, spin shortly;
5. Put 1,1mL of reaction buffer in a clean 1.5mL tube, add
   - 11µL of **"Cysteine",**
   - 11µL of **"NAD"**
   - 22µL of **"IMPDH enzyme"** solution,

   Do not add **ITP** solution
6. Close, agitate by inverting, spinning down briefly to ensure all the liquid is collected at the bottom of the tube.

V. Hemolysates preparation

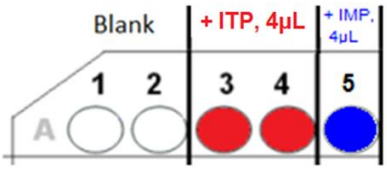
This protocol was developed with erythrocytes purified from 1mL of peripheral blood. The pellet of PBS-washed packed erythrocytes (from 1ml of blood) was resuspended in 4mL of ice-cold dH_20 and sonicated for 1min on ice (Sonopuls, Bandelin, 20% cycle, 50% power). The sonicated hemolysate was immediately used for ITPase measurement. The hemolysates can be also prepared by freeze-thawing of erythrocytes resuspended in water and high-speed centrifugation. Since the efficiency of hemolysis and release of ITPase enzyme depends on the method used for RBC disruption, we recommend to use always the same protocol of hemolysate preparation.

VI. Reaction monitoring for hemolysates

1. Program plate reader for kinetics absorbance reading (every 1 min), 30-37°C*

   **Optimal temperature for enzyme activity can vary significantly depending on the source and should be determined individually;*

   ***33°C was found as optimal temperature for human hemolysates.*



2. Add 4µL of hemolysates (or 1-10mUnits of ITPase from other source) per well to four wells, followed by addition of 200µL of **"Reaction mixture"**

   Recommended: *sometimes microplate agitation is insufficient and can result in an inhomogeneous solution, which can cause signal fluctuations during incubation. To address this, you might consider manual mixing: add 17µL of hemolysate to 0.85ml of reaction buffer, agitate by inverting, spin down shortly to recover all the liquid at the bottom of tube, dispense 200µL per well.*

3. **Positive control.** To assure that ITP-ase activity is not limited by IMPDH, add 200µL of complete reaction mixture to the well A5;
4. Insert the plate into the reader pre-heated at 33°C, agitate for 1 min and pre-incubate for 10 min;
5. To start the reaction, add 4µL of ITP solution to two wells (shown in red), two wells without ITP will be used as Blank.
6. Add 4µL of IMP solution to A5 well. Agitate for 1 min and monitor the reaction at 340nm at 33°C for 30 min with data collection every minute.

VII. Calculation of ITPase activity (U/ml)

Typical results obtained with RBC lysates (4µL per well, hemoglobin 1mg/ml) are shown on Table 1 / Figure 1.

1. Calculate the absorbance rate per min for reaction buffers with ITP (AR) and without (AR_{blank}) using "Slope" function of Excel.
2. Calculate ITPase activity in lysate (or other enzyme source) 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)

1 Unit (U) is defined as 1 µmol of product formed per min

Dilution factor = well volume (200µL) / added volume (4µL)

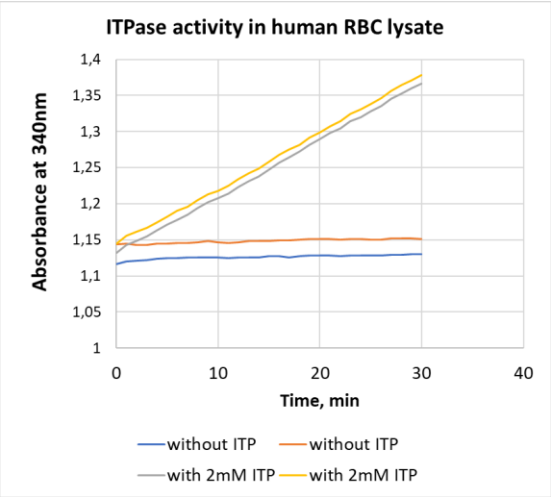


Figure 1. Kinetics of formation of IMP catalyzed by hemolysate ITPase in the absence and the presence of 2mM ITP. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 33°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797).

Table 1.

Time, min	without ITP	with 2mM ITP	without ITP	with 2mM ITP	IMP
0	1,116	**1,132**	1,144	**1,145**	0,812
1	1,12	**1,143**	1,145	**1,156**	1,261
2	1,121	**1,148**	1,143	**1,161**	1,626
3	1,122	**1,155**	1,143	**1,167**	1,941
4	1,124	**1,163**	1,145	**1,174**	2,213
5	1,125	**1,171**	1,145	**1,182**	2,449
6	1,125	**1,178**	1,146	**1,19**	2,646
7	1,126	**1,185**	1,146	**1,196**	2,826
8	1,126	**1,194**	1,147	**1,205**	2,963
9	1,126	**1,202**	1,148	**1,213**	3,073
10	1,126	**1,208**	1,147	**1,218**	Saturation
11	1,125	**1,214**	1,146	**1,225**	Saturation
12	1,126	**1,223**	1,147	**1,234**	Saturation
13	1,126	**1,231**	1,148	**1,242**	Saturation
14	1,126	**1,238**	1,148	**1,249**	Saturation
15	1,127	**1,247**	1,148	**1,258**	Saturation
16	1,127	**1,257**	1,149	**1,268**	Saturation
17	1,126	**1,264**	1,149	**1,275**	Saturation
18	1,127	**1,272**	1,15	**1,282**	Saturation
19	1,128	**1,282**	1,151	**1,292**	Saturation
20	1,128	**1,29**	1,151	**1,299**	Saturation
21	1,128	**1,298**	1,151	**1,307**	Saturation
22	1,127	**1,304**	1,15	**1,314**	Saturation
23	1,128	**1,314**	1,151	**1,324**	Saturation
24	1,128	**1,32**	1,151	**1,331**	Saturation
25	1,128	**1,328**	1,15	**1,338**	Saturation
26	1,128	**1,335**	1,15	**1,346**	Saturation
27	1,129	**1,345**	1,152	**1,356**	Saturation
28	1,129	**1,353**	1,152	**1,365**	Saturation
29	1,13	**1,36**	1,152	**1,371**	Saturation
30	1,13	**1,366**	1,151	**1,378**	Saturation
Absorbance rate per min (AR, AU/min)	0,0003	0,0079	0,0003	0,0078	
Absorbance rate after Blank subtraction (ARblank, AU/min)	0,0076		0,0075		
ITPase activit	0,0439		0,0436		