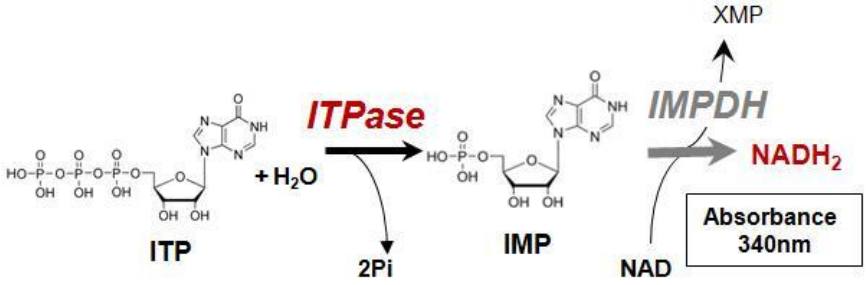
Continuous PRECICE® ITP pyrophosphohydrolase Assay Kit

For a one-step enzymatic measurement of inosine triphosphate pyrophosphohydrolase (ITPA, ITPase)

I. Introduction

PRECICE® ITPase Assay Kit is the first non-radioactive and continuous kit designed to measure ITP pryophosphohydrolase content in samples. This enzymatic assay is based on a reaction involving Inosine Monophosphate Dehydrogenase (IMPDH).

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



(1) In the presence of ITP, **ITP pyrophosphohydrolase** enzyme catalyzes the formation of IMP

(2) In the presence of NAD, 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 with cell lysates 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 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 to perform 24 analyses in a time (8 samples in triplicate, 12 samples in duplicate).

A standard PRECICE® ITPase Assay Kit contains:

- one tube **"Cofactor 1"**
- one tube **"Cofactor 2 "**
- 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 ITP"** blue cap (pre-filled with 5µmol ITP);
- one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

V. 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", "Cofactor 2", 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 a 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.

2. Transfer 5ml of complete "Reaction mixture 1x" containing enzymes and cofactors to blue cap 15ml tube pre-filled with ITP**.**

You have prepared: 5ml of **"Blank"**
5ml of **"Reaction mixture with 1mM ITP"**

VI. Microplate preparation

1. **Preparation of hemolysates.** The pellet of PBS-washed erythrocytes from 100µL of blood was frozen-thawed twice, resuspended in 500µL of ice-cold deionized water and used directly for ITPase quantification.

2. Add 5µL of hemolysates (indicated as S1-S11) per well as shown below:

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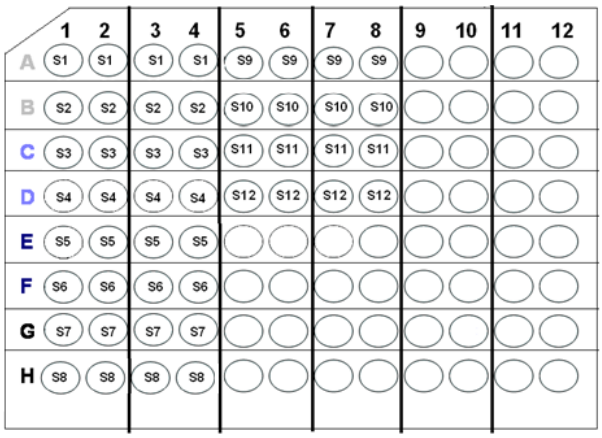

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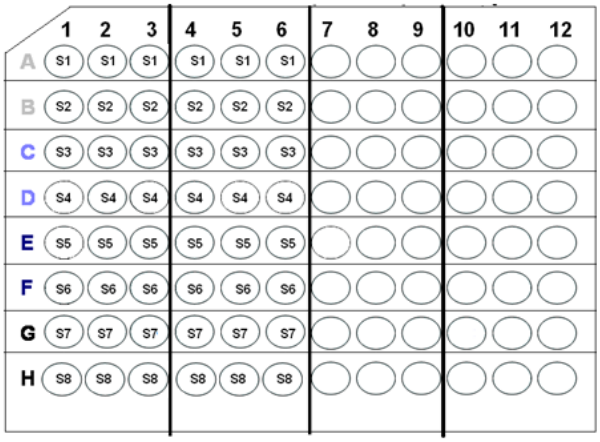

3. Add 200µL of "Blank" per well and 200µL of **"Reaction mixture"** containing **1mM ITP** as shown below:

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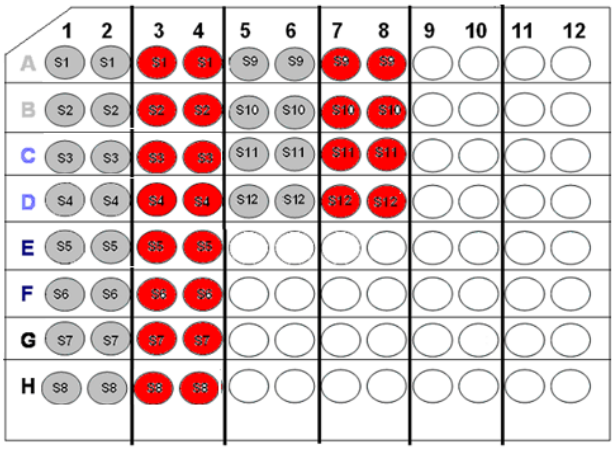

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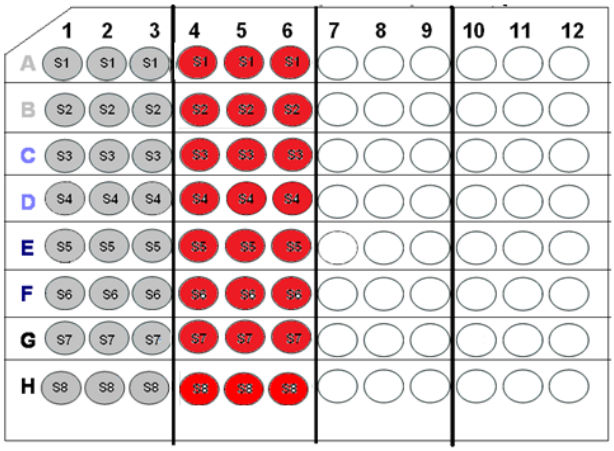

4. Program plate reader for kinetics absorbance reading (every 2min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 1 hour with data collection every 2min. Typical results obtained with RBC lysates are shown on Table 1 / Figure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

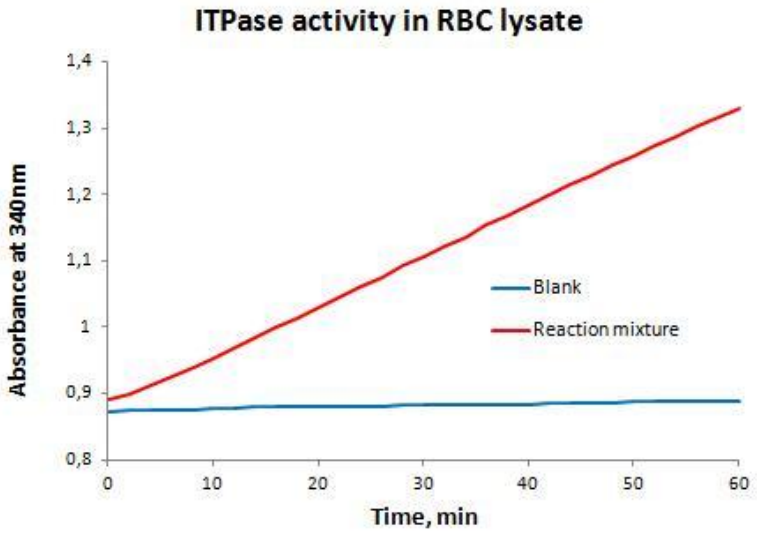

Figure1. Kinetics of formation of IMP catalyzed by ITP pyrophosphohydrolase in hemolysates in the absence and the presence of ITP. **After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref. 3797).**

Time, min	Blank		Reaction Mixture	
0	0,828	0,873	0,964	0,891
2	0,828	0,874	0,975	0,9
4	0,827	0,876	0,987	0,911
6	0,828	0,875	0,997	0,925
8	0,828	0,876	1,006	0,938
10	0,829	0,877	1,02	0,953
12	0,829	0,878	1,033	0,969
14	0,83	0,879	1,048	0,983
16	0,83	0,879	1,063	0,999
18	0,831	0,879	1,079	1,013
20	0,831	0,88	1,095	1,03
22	0,832	0,88	1,111	1,045
24	0,832	0,881	1,129	1,061
26	0,833	0,881	1,146	1,075
28	0,833	0,882	1,164	1,092
30	0,834	0,882	1,181	1,106
32	0,834	0,882	1,197	1,123
34	0,835	0,883	1,214	1,136
36	0,835	0,884	1,231	1,153
38	0,836	0,884	1,247	1,167
40	0,836	0,884	1,264	1,183
42	0,838	0,885	1,28	1,199
44	0,837	0,886	1,296	1,214
46	0,837	0,886	1,312	1,229
48	0,838	0,886	1,327	1,244
50	0,838	0,887	1,344	1,259
52	0,838	0,887	1,359	1,273
54	0,839	0,888	1,375	1,288
56	0,839	0,888	1,391	1,303
58	0,839	0,888	1,406	1,316
60	0,84	0,889	1,421	1,33
Absorbance rate per minute	0,0002228	0,0002482	0,007915121	0,007530645
Absorbance rate per hour	0,0133669	0,0148911	0,474907258	0,45183871
ITPase activity in nmol/hour/ml			94,03637261	89,3285056

VI. Calculation of ITPase activity in hemolysates

1. Calculate the absorbance rate per hour for reaction buffers with ITP (AR_{ITP}) and without (AR_{blank}).

2. Calculate Mean AR_{ITP} 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. ITPase activity is calculated by the following formula:

$$\text{Activity} = \frac{\text{Mean } AR_{ITP} - \text{Mean } AR_{blank}}{4.9 \times [\text{Hgb}]} \times 10^3 = \frac{(0.463 - 0.014)}{4.9 \times 0.97} \times 10^3 = 94.5 \text{ nmol/ hour / mg of Hgb}$$

Where: Mean $AR_{ITP} = 0.463$

Mean $AR_{blank} = 0.014$

[Hgb], final haemoglobin concentration used in assay = 0.97 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).