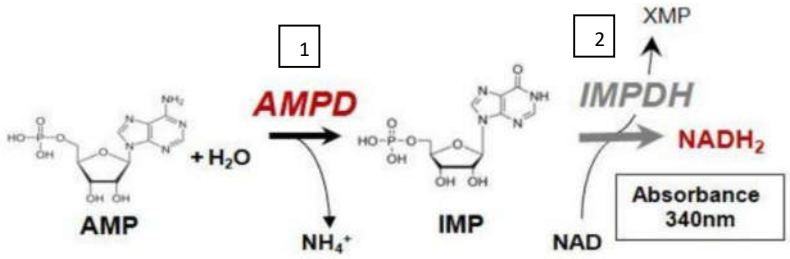
Continuous PRECICE® AMP Deaminase Assay Kit: User manual - Ref: # K0709-05-2

For measurement of 5'-adenosine monophosphate deaminase (AMPD, AMPDA) activity

I. Introduction

PRECICE® AMPD Assay Kit is the first non-radioactive and continuous assay designed to measure AMP-deaminase 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 AMP, AMP Deaminase (AMPD) 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 AMP deaminase activity *in vitro* or in cell lysates. *For maximal accuracy,* the assays with cell lysates are run **with and without AMP substrate** 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 AMPD enzyme, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® AMPD 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 provided in the kit are not stable and should be stored on ice and used the day of preparation. The kit allows performing 24 analyses in a time (12 samples in duplicate or 8 samples in triplicate).

A standard PRECICE® AMPD Assay Kit contains:

- **one tube "Cofactors" (DTT and NAD);**
- **one tube "IMPDH enzyme";**
- **one glass vial "Blank" (pre-filled with 10mL of reaction buffer);**
- **one 15mL tube "Reaction mixture with AMP (25µmol)";**
- **one transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)**

The kit is shipped at room temperature since 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.

NovoCIB SAS, 2 rue Solferino 62200 Boulogne-sur-Mer, France contact@novocib.comTel / Fax +33 (0)3 91 18 70 23

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 37°C**;**
3. Add 200µL of deionized water to the tube with "**IMPDH enzyme",** agitate (do not vortex to avoid foam;
4. Quantitatively transfer **"Cofactors"** to **"Blank"** tube

   To do so:

   - Add pipet 1ml of buffer from **"Blank"** to the tube, mix by inverting until the powder is dissolved.

   - transfer the content back into a vial **"Blank"** by pipeting.

   - repeat to be sure that all cofactors are recovered. Mix by inverting;
5. Add dissolved **"IMPDH enzyme"** to **"Blank"**; close and agitate by inverting, spin shortly;
6. Transfer 5ml of complete "Reaction mixture" containing enzymes and cofactors to orange cap 15ml tube pre-filled with AMP (powder). You have prepared:
   5ml of **"Blank"**
   5ml of **"Reaction mixture with AMP"**
   Equilibrate both solutions at 37°C**.**

V. Preparation of hemolysates

This protocol was developed with erythrocytes purified from 1mL of peripheral blood using Ficoll-Hypaque gradient and washed once with PBS.

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 hemolysates were immediately used for HPRT measurement without additional centrifugation.

The hemolysates can be also prepared by numerous freeze-thawing of erythrocytes resuspended in water and high speed centrifugation. Since the efficiency of hemolysis and release of HPRT enzyme depends on the method used for RBC disruption, we recommend to use always the same protocol of hemolysate preparation.

VI. Microplate preparation

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

2. Add 4µL of hemolysates (indicated as S1-S11) per well as shown below:

3. Add 200µL of **"Blank"** per well and 200µL of **"Reaction mixture with AMP"** as shown below:

4. Program plate reader for kinetics absorbance reading (every 1 min), 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 30min-1 hour. Typical results obtained with RBC lysates are shown on Table 1 / Figure 1.

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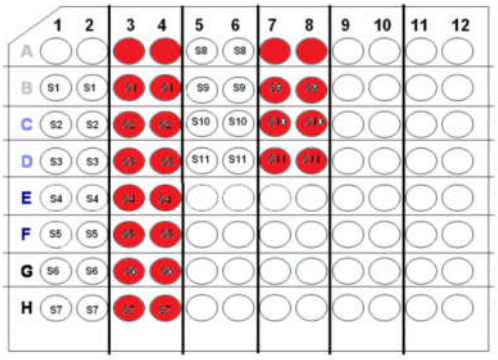

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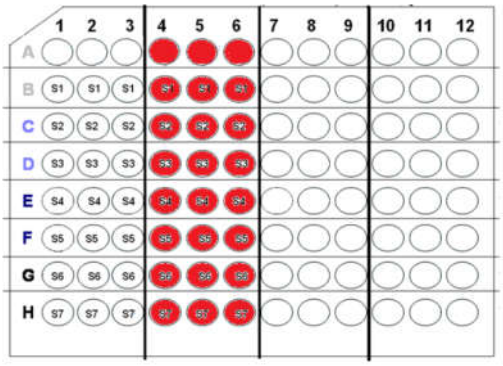

VII. **Calculation of activity of AMP-DA**

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

2. Calculate Mean AR_{AMP} 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. AMPD activity is calculated by the following formula:

$$\text{Activity} = \frac{\text{Mean } AR_{AMP} - \text{Mean } AR_{blank}}{4.9 \times [\text{Hgb}]} \times 10^3 = \frac{(0.948 - 0.023)}{4.9 \times 0.95} \times 10^3 = 199 \text{ nmol/ hour / mg of Hgb}$$

Where: Mean AR_{AMP} = 0.948

Mean AR_{blank} = 0.023

[Hgb], final haemoglobin concentration used in assay = 0.95 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).

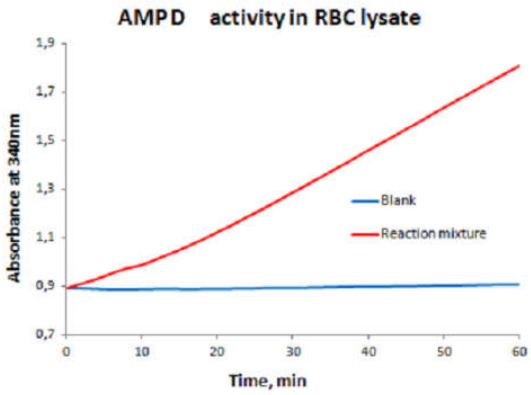

Figure 1. Kinetics of formation of IMP catalyzed by AMP demainase S in hemolysates in the absence and the presence of AMP. After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®,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

Time, min	Blank		Reaction mixture	
0	0,893	0,892	0,921	0,893
2	0,892	0,891	0,93	0,913
4	0,89	0,89	0,944	0,932
6	0,888	0,892	0,961	0,955
8	0,888	0,891	0,981	0,976
10	0,889	0,893	1,002	0,99
12	0,89	0,893	1,025	1,014
14	0,891	0,895	1,052	1,039
16	0,889	0,896	1,079	1,065
18	0,89	0,898	1,108	1,094
20	0,89	0,899	1,139	1,124
22	0,891	0,902	1,17	1,155
24	0,891	0,901	1,202	1,188
26	0,893	0,903	1,235	1,22
28	0,893	0,904	1,268	1,254
30	0,893	0,904	1,302	1,288
32	0,894	0,905	1,336	1,322
34	0,895	0,907	1,371	1,357
36	0,896	0,908	1,405	1,391
38	0,897	0,909	1,441	1,426
40	0,897	0,91	1,476	1,462
42	0,897	0,912	1,51	1,496
44	0,899	0,912	1,545	1,531
46	0,899	0,913	1,58	1,566
48	0,9	0,914	1,616	1,601
50	0,901	0,915	1,651	1,637
52	0,901	0,916	1,686	1,671
54	0,902	0,917	1,72	1,706
56	0,903	0,918	1,756	1,741
58	0,904	0,919	1,79	1,775
60	0,904	0,919	1,824	1,81
Absorbance rate per minute	0,0002577	0,0005238	0,0158123	0,0157994
Absorbance rate per hour	0,0154597	0,0314274	0,9487379	0,9479637
AMP-DA activity in nmol/hour/ml			188,83558	188,67758