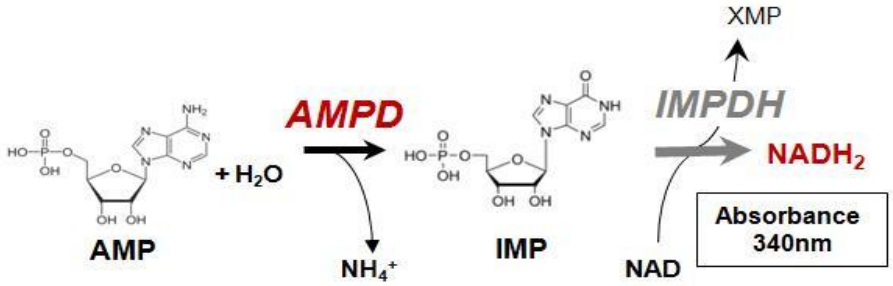
Continuous PRECICE® AMP Deaminase Assay Kit

For a one-step enzymatic measurement of 5'-adenosine monophosphate deaminase (AMPDA, AMPD)

I. Introduction

PRECICE® AMPD Assay Kit is the first non-radioactive and continuous kit 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** 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 AMPDA activity *in vitro* or in cell lysates.

For maximal accuracy, the assays with cell lysates are run **with and without AMP** in parallel. The absorbance rate observed in the absence of AMP 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 AMPDA 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 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® AMP Deaminase 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 AMP"** blue cap (pre-filled w 25µmol AMP);
- 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 AMP**.**

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

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 AMPDA quantification.

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

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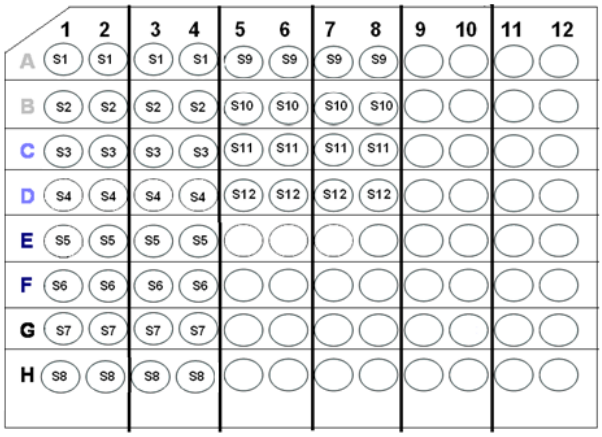

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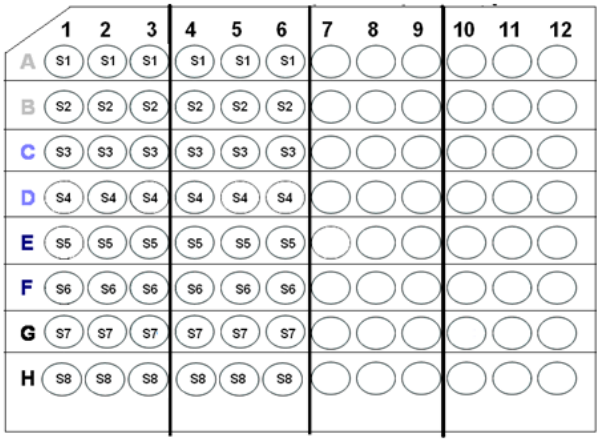

3. Add 200µL of "Blank" per well and 200µL of **"Reaction mixture"** containing **5mM AMP** as shown below:

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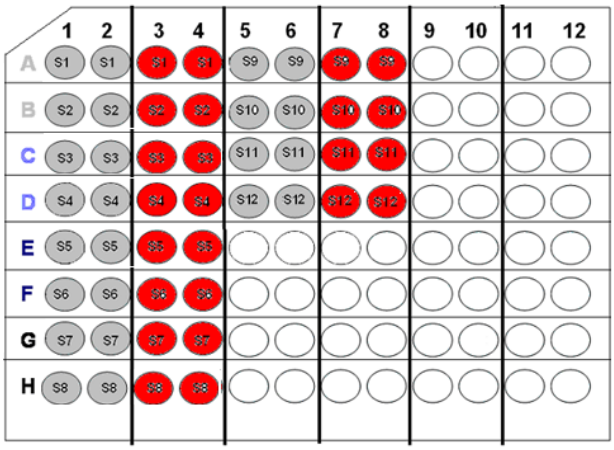

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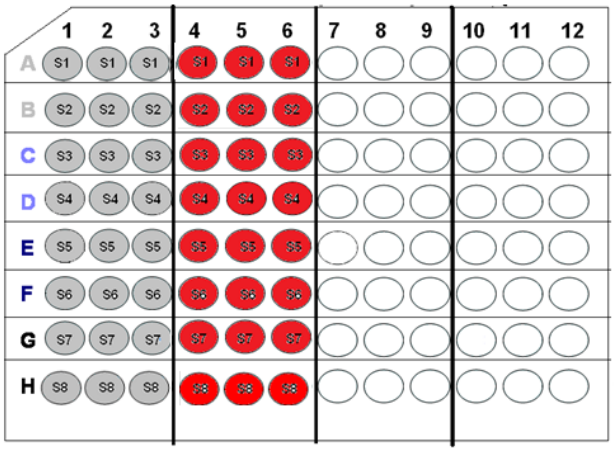

4. Program plate reader for kinetics absorbance reading (every 2min), 37°C.
Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 1 hour with data collection every 2min. Typical results obtained with RBC lysates are shown on Table 1 / Figure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

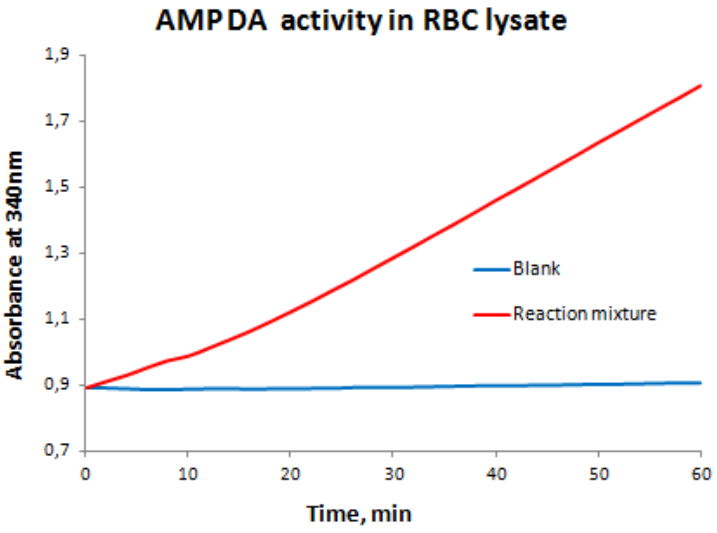

Figure1. Kinetics of formation of IMP catalyzed by AMP deaminase in hemolysates in the absence and the presence of AMP. **After vigorous shaking for 1min, the absorbance at 340nm was monitored at 37°C using iEMS Plate Reader (Thermo Scientific) and round-bottom 96-well microplate (Corning, Costar®, ref.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

VI. Calculation of AMPD activity in hemolysates

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).