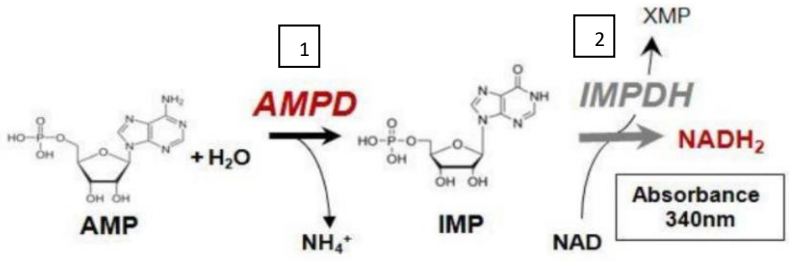
Continuous PRECICE® AMP Deaminase Assay Kit:
User manual - Ref: # K0709-05-2

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 contains 2 sets of tubes for preparing 2x5mL of reaction mixture:

Once dissolved, the reagents (5ml of reaction mixture containing DTT, NAD and IMPDH) are stable for 2-3 days if stored at +4 - +8°C. This mixture should be always warm up before the experiment.

Each set contains:

- **Cofactor 1** (powder);
- **Cofactor 2** (powder);
- **AMP** (powder);
- **IMPDH enzyme, lyophilized;**
- **Reaction buffer** (glass vials, 5mL);
- **IMP** (positive control for IMPDH)
- **Purified bacterial AMPD-deaminase** (AMPDA, positive control)
- **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.

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IV. Preparation of 5mL of "Reaction mixture"

IMPORTANT: Use only autoclaved Milli-Q water to inactivate ubiquitous phosphatases and to avoid nucleootides dephosphorylation

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. Quantitatively transfer **"Cofactor 1", "Cofactor 2"** and **"IMPDH"** to 5mL of **"Reaction buffer"**.

   To do so:

   -pipet 1ml of **"Reaction buffer"** to each of 3 tubes and mix them by inverting or pipeting up and down until the powder dissolved.

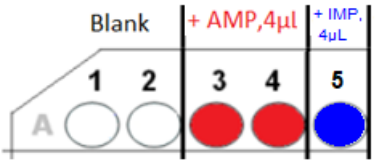
   - transfer by pipetting the content of three tubes back into a vial **"Reaction buffer"**.

   - 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.
4. Add 100µL of phosphatase-free water to AMP, agitate until complete dissolution of the powder;
5. Add 200µL of phosphatase-free water to IMP powder, agitate too dissolve;
6. Add 400µL of phosphatase-free water to lyophilized **AMPDA**, do not vortex immediately, leave for 10min to solubilise pellet, vortex vigorously and spin the tube to remove the foam.

VI. Reaction monitoring

1. Program plate reader for kinetics absorbance reading every 1min, 37°C;
2. **Positive control 1.** Add 400µL of deionized water to lyophilized AMPDA enzyme provided with the kit to produce 350mU/ml solution and mix gently until the powder is dissolved.

   Add 4µL of this AMPDA solution per well in four wells (A1-A4);
3. Add 4µL of AMPDA solution to be characterized to four wells **(suggested B1-B4, C1-C4, D1-D5, E1-E4, F1-F4)**, followed by addition of 200µL of **"Reaction mixture";**



4. **Positive control 2.** To assure that AMPDA activity is not limited by IMPDH, add 200µL of complete reaction mixture to the well A5;
5. Insert the plate into the reader pre-heated at 37°C, agitate for 1 min and incubate for 15 min;
6. To start the reaction, add 4µL of AMP solution to the wells of column 3 and 4, shown in red (two others will be used as Blank).
7. Add 4µL of IMP solution to A5 well. Agitate for 1 min and monitor the reaction at 340nm at 37°C for 30 min with data collection every minute.

   Typical results obtained with purified AMP-DA are shown on Table 1 / Figure 1.

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. AMPD activity (U/ml) in well is calculated by the following formula:
4. Activity (U/ml) = (Mean AR_{AMP} -Mean AR_{Blank})*50/4.9

Where 50 is a dilution factor (4µL per well of 200µL)

And 4.9 is the absorbance of 1mM NADH at 340nm in round-bottom well of Corning microplate Ref. 3797

IMPDH activity (0.150U/ml in well) should be always 3-10 times superior to AMPDA activity. If AMDA activity is close or comparable to IMPDH activity, dilute AMPDA sample and repeat the experiment.

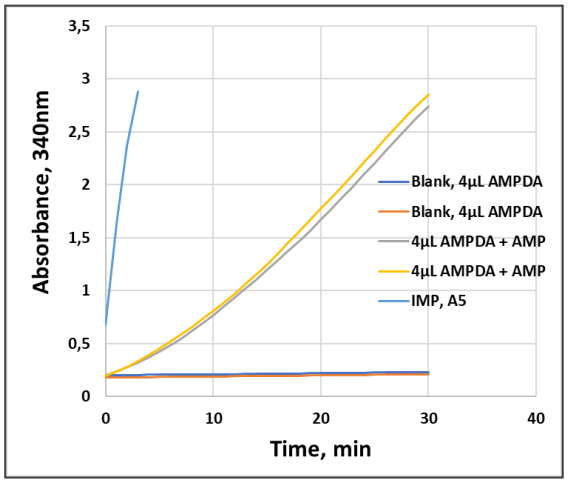


Figure 1. Kinetics of IMP formation from AMP catalysed by AMPDA provided with the kit (4µL per well).

Table 1.

Time, min	Blank, 4µL AMPDA	Blank, 4µL AMPDA	4µL AMPDA + AMP	4µL AMPDA + AMP	IMP, A5
0	0,202	0,181	0,2	0,197	0,676
1	0,203	0,182	0,233	0,233	1,634
2	0,204	0,182	0,275	0,281	2,371
3	0,204	0,183	0,321	0,335	2,88
4	0,205	0,183	0,371	0,392	saturated
5	0,205	0,184	0,424	0,453	saturated
6	0,206	0,185	0,482	0,518	saturated
7	0,206	0,186	0,547	0,585	saturated
8	0,207	0,187	0,618	0,656	saturated
9	0,208	0,188	0,692	0,731	saturated
10	0,209	0,189	0,77	0,808	saturated
11	0,21	0,19	0,85	0,889	saturated
12	0,211	0,191	0,933	0,973	saturated
13	0,212	0,192	1,018	1,06	saturated
14	0,213	0,193	1,104	1,151	saturated
15	0,215	0,194	1,193	1,246	saturated
16	0,215	0,194	1,283	1,345	saturated
17	0,217	0,196	1,375	1,453	saturated
18	0,217	0,196	1,468	1,56	saturated
19	0,22	0,199	1,568	1,671	saturated
20	0,22	0,199	1,671	1,777	saturated
21	0,222	0,201	1,776	1,88	saturated
22	0,223	0,201	1,879	1,986	saturated
23	0,225	0,203	1,987	2,098	saturated
24	0,224	0,203	2,095	2,207	saturated
25	0,226	0,205	2,205	2,321	saturated
26	0,228	0,206	2,314	2,429	saturated
27	0,229	0,207	2,426	2,543	saturated
28	0,23	0,208	2,533	2,648	saturated
29	0,231	0,21	2,639	2,752	saturated
30	0,232	0,211	2,742	2,854	saturated
Absorbance rate, AU/min	**0,001**	**0,001**	**0,087**	**0,092**	**0,735**
Mean absorbance rate, AU/min	**0,001**		**0,090**		**0,735**
AMPDA activity in well, U/ml			**0,018**		**0,150**
AMPDA activity in stock solution, U/ml			**0,882**		