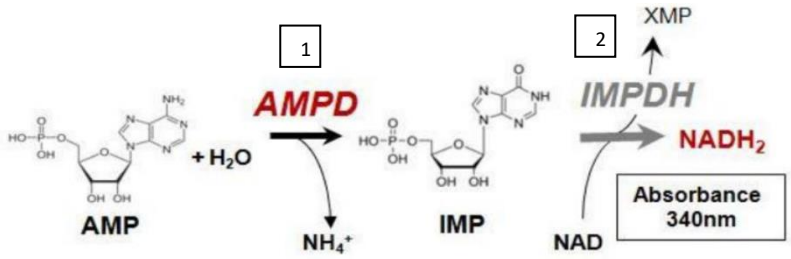
Continuous PRECICE® AMP Deaminase Assay Kit:
User manual - *Ref: # K0709-05-1*

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 1 set of tubes to prepare 5mL of reaction mixture:

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.

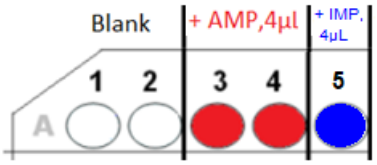
IV. Preparation of 5mL of "Reaction mixture"

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 room temperature**;**
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 pipetting 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 100µL of phosphatase-free water to IMP powder, agitate too dissolve;
6. Add 200µL of phosphatase-free water to lyophilized **AMP-DA**, do not vortex immediately, leave for 10min to solubilise pellet, vortex vigorously and spin the tube to remove the foam.

VI. Assay of purified or partially purified AMP-DA



1. Program plate reader for kinetics absorbance reading every 1min, 37°C;
2. **Positive control 1.** 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 AMP-DA 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.
8. Insert microplate, agitate and monitor the reaction at 340nm at 37°C for 30 min or longer with data collection every minute. Typical results obtained with RBC lysates are shown on Figure 1 and Table 1.
9. To calculate the activity of AMP-DA, paste the data into **Excel table** available at contact@novocib.com upon request or follow the instructions below.

VII. Calculating the activity of AMP-deaminase (U/ml)

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:

$$\textbf{Activity (U/ml)} = (\text{Mean } AR_{AMP} - \text{Mean } AR_{Blank}) * 50/4.9$$

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

IMPORTANT: IMPDH activity (0.150U/ml in well) should be always 2-5 times superior to AMPDA activity. If AMP-DA activity is close or comparable to IMPDH activity, dilute AMP-DA sample and repeat the experiment.

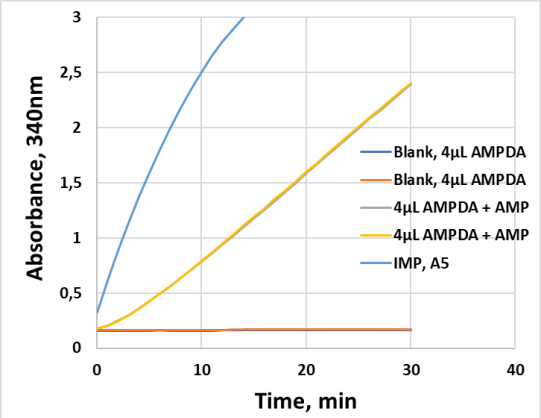


Figure 1. Kinetics of IMP formation from AMP catalysed by AMP-DA provided with the kit (4µL per well).

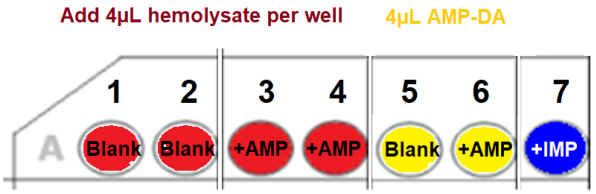
Table 1.

Time, min	Blank, 4µL AMPDA	Blank, 4µL AMPDA	4µL AMPDA + AMP	4µL AMPDA + AMP	IMP, A5
0	0,162	0,167	0,18	0,176	0,327
1	0,162	0,166	0,206	0,203	0,62
2	0,162	0,166	0,249	0,245	0,889
3	0,162	0,167	0,301	0,298	1,139
4	0,162	0,167	0,36	0,359	1,373
5	0,162	0,167	0,425	0,425	1,594
6	0,163	0,167	0,493	0,494	1,802
7	0,162	0,167	0,563	0,566	1,997
8	0,162	0,167	0,636	0,64	2,181
9	0,162	0,167	0,71	0,715	2,351
10	0,162	0,167	0,785	0,792	2,507
11	0,162	0,167	0,862	0,869	saturated
12	0,163	0,167	0,939	0,948	saturated
13	0,163	0,168	1,017	1,028	saturated
14	0,163	0,168	1,095	1,109	saturated
15	0,163	0,168	1,176	1,19	saturated
16	0,163	0,168	1,256	1,27	saturated
17	0,163	0,168	1,336	1,352	saturated
18	0,163	0,168	1,418	1,432	saturated
19	0,164	0,168	1,499	1,513	saturated
20	0,163	0,169	1,582	1,595	saturated
21	0,163	0,168	1,664	1,676	saturated
22	0,164	0,168	1,747	1,759	saturated
23	0,164	0,169	1,829	1,842	saturated
24	0,164	0,169	1,912	1,924	saturated
25	0,164	0,169	1,993	2,006	saturated
26	0,164	0,169	2,076	2,087	saturated
27	0,164	0,169	2,154	2,168	saturated
28	0,164	0,169	2,235	2,249	saturated
29	0,164	0,169	2,317	2,331	saturated
30	0,164	0,169	2,394	2,406	saturated
Absorbance rate, AU/min	**0,000**	**0,000**	**0,077**	**0,077**	**0,271**
Mean absorbance rate, AU/min	**0,000**		**0,077**		**0,271**
AMPDA activity in well, U/ml			**0,016**		**0,055**
AMPDA activity in stock solution, U/ml			**0,783**		

VIII. Assay of AMP-deaminase in cell lysates

This protocol was developed with erythrocytes purified from 1mL of peripheral blood. The pellet of PBS-washed packed erythrocytes (from 1ml of blood) was frozen resuspended in 4.5mL of ice-cold dH_20 and sonicated for 1min on ice (Sonopuls, Bandelin, 20% cycle, 50% power). The sonicated hemolysates (protein concentration 40mg/ml) were used for AMP-DA measurement. The hemolysates can be also prepared by several freeze-thawing of erythrocytes resuspended in water and high-speed centrifugation. Since the efficiency of hemolysis and release of AMP-DA enzyme depends on the method used for RBC disruption, we recommend to use always the same protocol of hemolysate preparation.

IX. Reaction monitoring



1. Program plate reader for kinetics absorbance reading every 1min, 37°C;
2. Add 4µL of hemolysates to wells A1, A2, A3 and A4, followed by addition of 200µL of complete **"Reaction mixture";**
3. **Positive control 1 (optional).** Add 4µL of prepared AMP-DA solution per well in four wells (A5-A6);
4. **Positive control 2 (optional).** Add 200µL of complete reaction mixture to the well A7;
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 a shown (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.

8. Insert microplate, agitate and monitor the reaction at 340nm at 37°C for 1h or longer with data collection every minute. Typical results obtained with RBC lysates are shown on Table 2 / Figure 2.

9. To calculate the activity of AMP-DA, paste the data into **Excel table** available at contact@novocib.com upon request or follow the instructions below.

Table 2.

Time, min	Blanc, 4µL RBC lysate	Blanc, 4µL RBC lysate	**4µL RBC lysate + AMP**	**4µL RBC lysate + AMP**	AMPDA, sans AMP	**AMPDA, + AMP**	**IMP**
0	0,781	0,824	**0,824**	**0,852**	0,188	**0,204**	**0,578**
1	0,783	0,826	**0,83**	**0,858**	0,189	**0,231**	**1,295**
2	0,783	0,826	**0,835**	**0,862**	0,19	**0,264**	**1,909**
3	0,784	0,829	**0,84**	**0,868**	0,191	**0,301**	**2,427**
4	0,785	0,828	**0,845**	**0,873**	0,191	**0,348**	**2,827**
5	0,787	0,83	**0,852**	**0,88**	0,192	**0,406**	**3,103**
6	0,787	0,831	**0,857**	**0,886**	0,193	**0,465**	**3,268**
7	0,789	0,831	**0,861**	**0,89**	0,194	**0,521**	**3,334**
8	0,789	0,833	**0,867**	**0,895**	0,195	**0,573**	**3,395**
9	0,791	0,834	**0,873**	**0,901**	0,196	**0,629**	**3,422**
10	0,79	0,833	**0,877**	**0,905**	0,197	**0,69**	**3,446**
11	0,792	0,836	**0,885**	**0,912**	0,198	**0,755**	**3,446**
12	0,792	0,836	**0,89**	**0,917**	0,199	**0,825**	**3,446**
13	0,793	0,836	**0,895**	**0,923**	0,2	**0,895**	**3,446**
14	0,794	0,838	**0,902**	**0,929**	0,202	**0,973**	**3,446**
15	0,794	0,838	**0,908**	**0,935**	0,203	**1,053**	**3,446**
16	0,793	0,837	**0,912**	**0,939**	0,203	**1,135**	**3,446**
17	0,795	0,839	**0,92**	**0,946**	0,205	**1,221**	**3,446**
18	0,796	0,84	**0,926**	**0,953**	0,206	**1,308**	**3,446**
19	0,796	0,84	**0,932**	**0,959**	0,207	**1,399**	**3,446**
20	0,796	0,84	**0,94**	**0,967**	0,208	**1,492**	**3,446**
21	0,797	0,841	**0,947**	**0,974**	0,208	**1,586**	**3,446**
22	0,798	0,842	**0,954**	**0,981**	0,21	**1,683**	**3,446**
23	0,798	0,842	**0,961**	**0,988**	0,21	**1,78**	**3,446**
Absorbance rate per minute	0,0007	0,0008	**0,0059**	**0,0058**	0,0010	**0,0692**	**0,5068**
Mean absorbance rate per min	0,001		**0,006**		0,0010	**0,0692**	**0,5068**
NADH formation rate, µmols/hour/mL	8,995		**71,43**				
Protein concentration in well, mg/ml	0,000		**0,800**				
AMP-DA activity in RBC lysate, nmol/hour/mg			**89,29**				

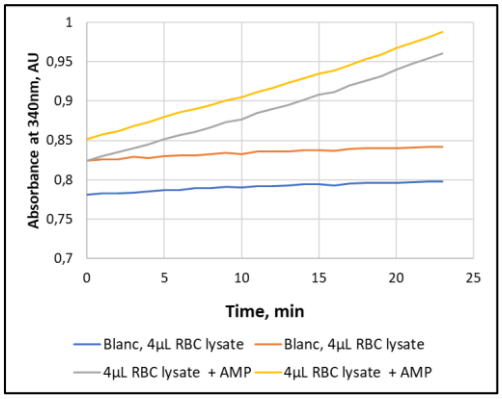


Figure 2. Kinetics of IMP formation from AMP catalysed by AMP-deaminase from hemolysate (4µL of hemolysate per well, 37°C).

X. Calculating the AMP-DA activity in cell lysate (nmols/hour/mg)

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 protein in hemolysates.

4. AMP-DA activity is calculated by the following formula:

Activity (nmols/hour/mg of protein) = (Mean AR_{AMP} -Mean AR_{Blank})*10^3/4.9 /[protein]

Where 4.9 is the absorbance of 1mM NADH at 340nm in round-bottom well of Corning microplate Ref. 3797
[protein], final protein concentration in well