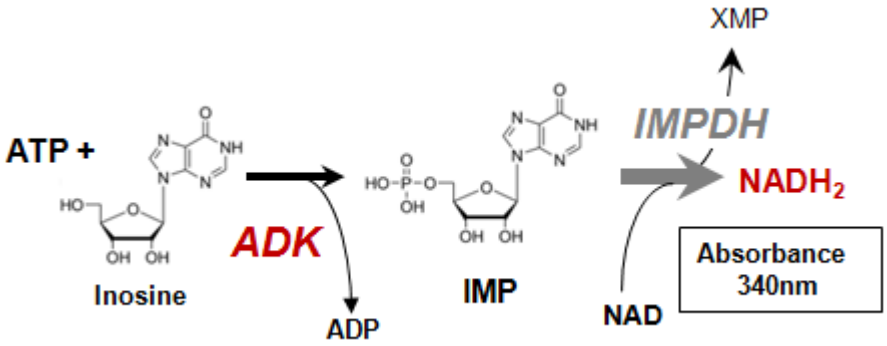
PRECICE® ADK Assay Kit

For research use only. Not for use in diagnostic procedures

I. Introduction

Once released outside the cell, adenosine, a naturally occurring ribonucleoside, reveals strong neuroprotective and anti-inflammatory properties. One of the strategies to increase extracellular adenosine consists in inhibiting adenosine kinase (ADK), a ubiquitous enzyme that catalyzes the transfer of γ-phosphate from ATP to the 5'-hydroxyl of adenosine generating AMP and ADP.

PRECICE® ADK Assay Kit is a non isotopic assay that allows a continuous monitoring of ADK activity at 340nm through the coupling of ADK-catalyzed phosphorylation of inosine to the oxidation of IMP by a recombinant IMP-dehydrogenase (IMPDH). The assay developed in microplate format allows a HTS search of novel ADK inhibitors.



PRECICE® ADK Assay Kit was validated with a known ADK inhibitor (A-134974, IC50=20nM) and meets the requirements of a convenient and reliable HTS assay (microplate format, "add-and measure", spectrophotometric continuous readout, Z-factor = 0.68).

ADK Assay Kit can be also used to evaluate the phosphorylation of novel nucleoside analogues. In the absence of nucleoside competitor, adenosine kinase phosphorylates inosine resulting in the formation of IMP directly monitored by the formation kinetics of NADH2 catalyzed by IMP-dehydrogenase. In the presence of nucleoside competitor, the phosphorylation of inosine, poor ADK substrate, is inhibited and detected as a decrease in NADH2 formation.

This user manual gives the instructions for standard assays in 96-well plate.

II. Kit Contents

A standard **PRECICE® ADK Assay Kit** (one 96-well plate) contains:

1. one tube **"IMPDH "**
2. one tube **"Cofactor 1"** (DTT)
3. one tube **"Cofactor 2"** (NAD)
4. one tube **"Human recombinant Adenosine Kinase"**
5. one tube **"ATP"**

6. one vial **"Reaction Buffer 5x"**
7. one tube **"ADK inhibitor"** (A-134974)
8. one tube **"50mM inosine"**
9. Transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

III. Equipments required

1. Plate agitator
2. Plate reader fitted with a filter 340nm.

IV. Storage

PRECICE® ADK Assay Kit must be **stored at -20°C** until used.

IMPORTANT:
The following instructions are given to measure the activity of ADK enzyme *in vitro*, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® ADK Phosphorylation 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.

V. Experimental Protocols

V.1. Microplate preparation.

i) To assess the performance of the assay, it is strongly recommended to run, in parallel with the assays and on the same microplate, several controls:

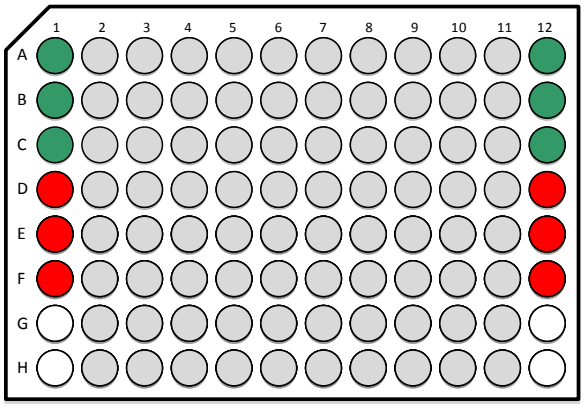
- **Positive controls**, add 2µL of 50µM A-134974 dissolved in DMSO per well.

A-134974 is a validated ADK inhibitor, commercialized by Sigma-Aldrich under licence of Abbott Laboratories

- **Negative controls**, 2µL of DMSO
- **Blank** assays, 2µL of DMSO, no inhibitor, but where the reaction was NOT started

Controls (in triplicate) and Blank Assays (in duplicate) can be set up in columns 1 and 12, as indicated on **Figure 2**.

ii) Add 2-5µL of a water or DMSO-dissolved compounds to be tested into the empty wells depicted as grey on **Figure 2**.



Positive controls 2µL 50µM A-134974

Negative controls 2µL DMSO

Assays

Blanks (2µL DMSO) / reaction not started

Figure 2: Example of the arrangement of positive and negative controls, blanks and assays on a 96-well microplate

VI. Experimental Protocol

*ADK enzyme is pre-icubated with the inhibitor in the presence of **ATP**, reaction started by **inosine** addition*

VI.1. Preparation of standard reaction buffer (1x)

i) Add the content of **"Reaction Buffer 5x"** vial (4ml) to 16ml of deionized water to prepare **"Reaction Buffer 1x"**.

ii) Transfer quantitatively the content of 3 tubes with "**Cofactor 1" (DTT), "Cofactor 2" (NAD), "ATP"** to the tube with **"Reaction buffer 1x"**.

To do so:

i) - transfer the content of three tubes (powder) into a vial **"Reaction buffer 1x";**

ii) – to be sure that all reagent and enzymes of the small tubes and vial are recovered, add 1 mL of **"Reaction buffer 1x"** to each empty tube, close, agitate and transfer the content back into the vial **"Reaction buffer 1x"**, mix by gently inverting until complete dissolution. Avoid bubbles.

iii) Solubilize the content of **"IMPDH"** tube by adding 1ml of **"Reaction buffer 1x"** with co-factors. Close, agitate and transfer the content tube back into a vial **"Reaction buffer 1x".**

iv) Solubilize the content of **"Human ADK enzyme"** tube by adding 1ml of complete **"Reaction buffer 1x"** with co-factors and IMPDH, transfer by pipeting the content of the tube back into a vial **"Reaction buffer 1x".**

VI.2 Following ADK activity in vitro

VI.2.1. Pre-incubation phase (15')

i) Program the plate-reader in a kinetics mode with the measurements done every 1 minutes, absorbance at 340 nm, 37°C, agitation before the kinetics for 1 min, duration time 15min.

ii) Add 200µL of standard reaction buffer per well.

iii) Agitate and measure absorbance at 340nm (A_{340}). Record this first set of data.

VI.2.2. Start the reaction by Inosine addition and follow-up the reaction for 40min

i) Eject the plate from the plate-reader

ii) Program the plate-reader in a kinetics mode with the measurements done every 1 minutes, absorbance at 340 nm, 37°C, agitation before the kinetics for 1 min, duration time 40min.

iii) Start the reaction by adding 10µL* of 50mM of **"Inosine"** per well

iv) Place the plate in the plate-reader and start the measurements. Record second set of data.

Composition of complete reaction buffer: 100mM Tris-HCl pH 8.5, 250mM KCl, 10mM MgCl2, 2.5mM NAD, 2.75mM ATP, IMPDH 20mU/ml; human recombinant ADK 2.2mU/ml – reaction started by inosine (final concentration 2.5mM)*
** the concentration of inosine can be further optimized by testing 0.5-1.25mM concentrations corresponding to 2-5µLof 50mM inosine per well.*

Quality control: measurement of Z' Factor of ADK assay

Determine the absorbance rate per minute for every **negative controls** (AR_{C-}), **positive controls** (AR_{C+}) and **blanks** (AR_{Blank}).

Calculate AR_{C-} and AR_{C+}, respectively the means of AR_{C-} measurements and of AR_{C+} measurements.

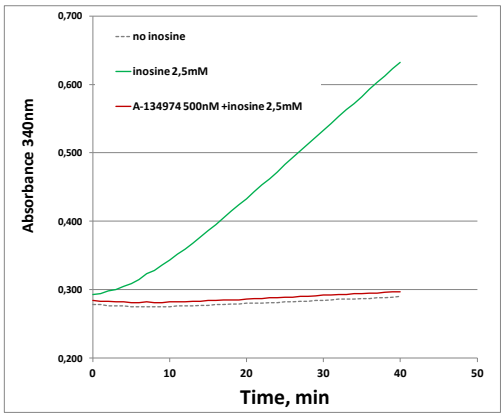
Calculate σ_{C-} and σ_{C+}, respectively the standard deviations of AR_{C-} measurements and of AR_{C+} measurements.

Z' Factor is then calculated as: $$Z' = 1 - \frac{(3\,\sigma_{c+} + 3\,\sigma_{c-})}{|\,\overline{AR_{C+}} - \overline{AR_{C-}}\,|}$$

Z' Factor value	Interpretation
1.0	Ideal
Between 0.5 and 1.0	Excellent assay
Between 0 and 0.5	Marginal assay
Less than 0	The signal from the positive and negative controls could overlap, making the assay not very useful or screening purposes

*Z-factor for ADK screening assay = 1- (3*0.0000594+3*0.000064)/I0.000523-0.009454/ = 0.958 -* ***excellent assay.***

Exemple of raw data:



			Negative control			Positive control		
	no inosine	no inosine	inosine 2,5mM	inosine 2,5mM	inosine 2,5mM	A-134974 500nM +inosine 2,5mM	A-134974 500nM +inosine 2,5mM	A-134974 500nM +inosine 2,5mM
0	0,278	0,283	**0,290**	**0,293**	**0,284**	**0,292**	**0,284**	**0,285**
1	0,278	0,282	**0,291**	**0,294**	**0,285**	**0,292**	**0,283**	**0,283**
2	0,276	0,281	**0,294**	**0,298**	**0,287**	**0,292**	**0,283**	**0,283**
3	0,276	0,281	**0,297**	**0,300**	**0,292**	**0,293**	**0,282**	**0,282**
4	0,276	0,280	**0,302**	**0,305**	**0,295**	**0,293**	**0,282**	**0,281**
5	0,275	0,280	**0,307**	**0,309**	**0,300**	**0,293**	**0,281**	**0,281**
6	0,275	0,280	**0,312**	**0,315**	**0,306**	**0,293**	**0,281**	**0,281**
7	0,275	0,280	**0,319**	**0,323**	**0,312**	**0,294**	**0,282**	**0,281**
8	0,275	0,280	**0,325**	**0,328**	**0,319**	**0,294**	**0,281**	**0,281**
9	0,275	0,280	**0,332**	**0,336**	**0,326**	**0,294**	**0,281**	**0,281**
10	0,275	0,280	**0,340**	**0,343**	**0,333**	**0,294**	**0,282**	**0,281**
11	0,276	0,280	**0,347**	**0,352**	**0,342**	**0,295**	**0,282**	**0,282**
12	0,276	0,281	**0,356**	**0,359**	**0,350**	**0,295**	**0,282**	**0,282**
13	0,276	0,282	**0,365**	**0,368**	**0,358**	**0,295**	**0,283**	**0,282**
14	0,277	0,282	**0,373**	**0,377**	**0,368**	**0,296**	**0,283**	**0,283**
15	0,277	0,282	**0,381**	**0,386**	**0,376**	**0,296**	**0,284**	**0,284**
16	0,278	0,283	**0,390**	**0,395**	**0,385**	**0,296**	**0,284**	**0,284**
17	0,278	0,283	**0,399**	**0,405**	**0,395**	**0,297**	**0,285**	**0,285**
18	0,279	0,284	**0,409**	**0,415**	**0,405**	**0,297**	**0,285**	**0,285**
19	0,279	0,284	**0,418**	**0,424**	**0,414**	**0,298**	**0,285**	**0,285**
20	0,280	0,285	**0,428**	**0,433**	**0,423**	**0,298**	**0,286**	**0,286**
21	0,280	0,285	**0,438**	**0,443**	**0,433**	**0,299**	**0,287**	**0,286**
22	0,280	0,286	**0,447**	**0,453**	**0,443**	**0,299**	**0,287**	**0,287**
23	0,281	0,286	**0,457**	**0,462**	**0,453**	**0,300**	**0,288**	**0,287**
24	0,281	0,287	**0,467**	**0,472**	**0,463**	**0,300**	**0,288**	**0,288**
25	0,282	0,288	**0,477**	**0,483**	**0,472**	**0,301**	**0,289**	**0,289**
26	0,282	0,288	**0,487**	**0,493**	**0,482**	**0,302**	**0,289**	**0,289**
27	0,283	0,289	**0,497**	**0,503**	**0,493**	**0,303**	**0,290**	**0,290**
28	0,283	0,289	**0,507**	**0,513**	**0,502**	**0,303**	**0,290**	**0,290**
29	0,284	0,290	**0,517**	**0,523**	**0,513**	**0,304**	**0,291**	**0,291**
30	0,284	0,290	**0,527**	**0,533**	**0,523**	**0,305**	**0,292**	**0,291**
31	0,285	0,291	**0,537**	**0,543**	**0,532**	**0,306**	**0,292**	**0,292**
32	0,286	0,292	**0,547**	**0,553**	**0,543**	**0,307**	**0,293**	**0,292**
33	0,286	0,292	**0,556**	**0,563**	**0,553**	**0,308**	**0,293**	**0,293**
34	0,286	0,293	**0,566**	**0,572**	**0,563**	**0,309**	**0,294**	**0,294**
35	0,287	0,293	**0,576**	**0,582**	**0,573**	**0,310**	**0,294**	**0,294**
36	0,287	0,294	**0,587**	**0,593**	**0,583**	**0,311**	**0,295**	**0,294**
37	0,288	0,294	**0,596**	**0,603**	**0,593**	**0,311**	**0,295**	**0,295**
38	0,288	0,295	**0,606**	**0,612**	**0,604**	**0,312**	**0,296**	**0,296**
39	0,289	0,295	**0,617**	**0,623**	**0,614**	**0,313**	**0,297**	**0,296**
40	0,290	0,296	**0,627**	**0,632**	**0,625**	**0,313**	**0,297**	**0,297**
Absorbance Rate AU/min	**0,00044**	**0,00049**	**0,00938**	**0,00948**	**0,00949**	**0,00060**	**0,00049**	**0,00049**
AR, mean			0,009454			0,000526		
AR, SD			5,94E-05			6,41E-05		
			C+			**C-**		