PRECICE® ADK Screening Assay Kit

For research use only. Not for use in diagnostic procedures

I. Background

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.

II. Principle

PRECICE® ADK Screening Assay Kit provides an enzymatic tool for continuous spectrophotometric monitoring of ADK activity in a convenient 96-well plate format. The ability of ADK to phosphorylate inosine (INO) is used in this assay by coupling the formation of inosine monophosphate (IMP) to its oxidation to xanthosine monophosphate (XMP), this latter reaction being catalyzed by inosine monophosphate dehydrogenase (IMPDH) in the presence of NAD. $NADH_2$ formation is directly monitored by spectrophotometry at 340nm. (Fig. 1).

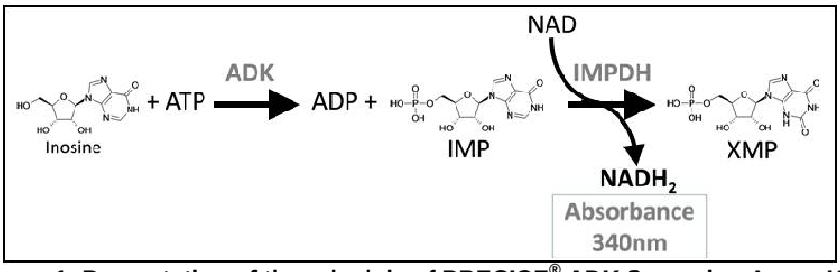

Figure 1: Presentation of the principle of PRECICE® ADK Screening Assay Kit.

PRECICE® ADK Screening Assay Kit has been developed for measuring ADK activity *in vitro*. Its use for the screening of ADK inhibitors and IC_{50} measurements was validated with compound A-134974, a known ADK inhibitor (Sigma-Aldrich, under license from Abbott Laboratories). Z'-Factor was measured from 40 negative (no inhibition) and positive (ADK inhibition with 500nM A-134974) controls. The assay showed a **Z'-Factor** of **0.68**, indicating an excellent assay performance for the screening of ADK inhibitors.

III. Kit Contents

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

- a vial of **"Recombinant IMPDH"** (~500mU with 0.250mg BSA)
- a vial of "**Human Recombinant ADK"** (~50mU)
- a vial of **"Enzyme Reconstitution Buffer"** (>500µL)
- a vial of **"NAD"** (27.86 mg, to be dissolved in 420 µL of deionized water)
- a vial of **"TKM Buffer 13.33x "** (1.575 mL)
- a vial of "**INO"** (28.2 mg to be dissolved in 1.05 mL of 0.1M KOH)
- a transparent microplate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

Are <u>not</u> provided:

- DTT (DL-dithiothreitol)
- ATP
- 0.1M KOH
- ADK inhibitor A-134974 (Sigma-Aldrich under licence from Abbot Laboratories, ref. A2846)

IV. Storage

PRECICE® ADK Screening Assay Kit must be **stored at -20℃** until used. Once enzyme or reagent solutions are reconstituted, they will be stored at **-20℃** , except NAD solution which will be preferentially stored at **-80℃** .

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 Screening 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

1. Reconstitution of IMPDH enzyme

Immediately prior to prepare the standard reaction buffer, reconstitute the lyophilized recombinant IMPH in 250µL of **"Enzyme Reconstitution Buffer"** and mix until dissolved to get a ~2U/mL solution.

This stock solution of reconstituted IMPDH is sufficient to prepare 21 mL of standard reaction buffer. For lesser volume (see below, V.3.), once the desired quantity of enzyme is pipetted for the preparation of standard reaction buffer, store the remaining stock solution of reconstituted IMPDH enzyme at -20℃.

2. Reconstitution of human recombinant ADK enzyme

Immediately prior to prepare the standard reaction buffer, reconstitute the lyophilized human recombinant ADK enzyme in 250µL of **"Enzyme Reconstitution Buffer"** and mix until dissolved to get a 200mU/mL solution.

This stock solution of reconstituted ADK contains more than enough mU of ADK to prepare 21 mL of standard reaction buffer (see below, V.3.). Once the desired quantity of enzyme is pipetted for the preparation of standard reaction buffer, store the remaining stock solution of reconstituted ADK enzyme at -20℃ .

3. Preparation of standard reaction buffer (1x)

Standard reaction buffer is prepared according to the following instructions.

For a 21mL total volume, which is sufficient for one 96-well plate:

i) Weight 16.17 mg of DTT in a clean tube labelled **"DTT"** and add 105 µL of dH_20 to prepare a 1M DTT solution. Mix until dissolved.
Important: ***DTT solution must be freshly prepared, since DTT in aqueous solution is highly unstable (half-life ~10h). Never use old solutions of DTT.***

ii) Weight 25.4 mg of ATP in a clean tube labelled **"ATP"** and add 420 µL of dH_20 to prepare a 0.1M ATP solution. Mix until dissolved.

iii) Prepare a 100 mM NAD solution by adding 420 µL of deionized water to **"NAD"** tube containing 27.86 mg of NAD powder. Mix until dissolved.

iv) In a tube containing 18.1 mL of dH_2O, transfer **in the following order** 1.575mL of **"TKM 13.33x"**, the content of vials **"DTT"**, **"NAD"**, **"ATP"** and **"Recombinant IMPDH"**. Add 130µL of vial **"Recombinant ADK"**

Alternatively, for other volume, prepare standard reaction buffer so that 1mL contain:

- 861.9 µL of dH_20
- 75 µL of "TKM 13.33x"
- 5 µL of 1M DTT
- 20 µL of 100mM NAD
- 20 µL of 100mM ATP
- 11.9 µL of recombinant IMPDH
- 6.2 µL of recombinant ADK

4. Preparation of INO solution

A 100 mM INO stock solution is prepared by adding 1050 µL of 100mM KOH solution to **"INO"** tube containing 28.16 mg of INO powder. Mix until dissolved. If needed, hold in hand to warm the tube up and facilitate dissolution.

5. Preparation of microplate and reading for ADK activity measurement *in vitro*

5.1. Preamble: Validation of the plate assay by measuring Z' factor with 500nM A-134974 used as an ADK inhibitor

5.1.1. Controls

To assess the performance of the assay, it is strongly suggested to run, in parallel with the assays and on the same microplate:

- Positive controls (ADK inhibition), by using compound A-134974 (a validated ADK inhibitor, Sigma-Aldrich under licence of Abbott Laboratories) at 500nM final concentration
- Negative controls (no ADK inhibition), where only DMSO is added into the wells
  N.B.: DMSO is added in negative control wells because it is the solvent of inhibitor A-134974 used in positive controls
- Blank assays, with no inhibitor, but where the reaction will not be started by adding INO

Control and Blank Assays can be set up in rows 1 and 2, as indicated on figure 2.

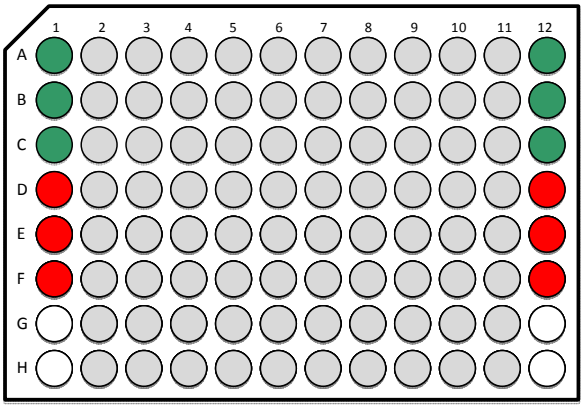

Figure 2: Example of the arrangement of positive and negative controls, blanks and assays on a 96-well microplate

5.1.2. Calculation of Z' Factor

ADK activities are measured for negative and positive controls as described in section 5.2. below. Please refer to it for measurement procedures and calculations of absorbance rates.

For each positive and negative control, determine the absorbance rate per minute for negative controls (AR_{C-}), positive controls (AR_{C+}) and blanks (AR_{Blank}).

Calculate $\overline{AR}_{C-}$ and $\overline{AR}_{C+}$, 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

5.2. ADK activity measurement

To assess the performance of the assay, it is strongly suggested to run, in parallel with the assays and on the same microplate, several controls. Please, refer to section 5.1. above for further details.

5.2.1. Pre-incubation phase (10')

i) Program the plate-reader in a kinetics mode, 340 nm, 37°C

ii) *For inhibition assay, pipette 2µL of a 100x concentrated solution* of the compounds to be tested into the wells.*
** will be diluted 100x in the well, after the addition of 200µL of standard reaction buffer*

iii) Add 200µL of standard reaction buffer into the wells.

iv) Pre-incubate in the plate-reader at 37°C and measur e Absorbance at 340nm (A_{340}) every 1 or 2 minutes for 10mn. A_{340} should stabilize during the pre-incubation phase. Record this first set of data.

5.2.2. Start the reaction and incubate (30-40')

i) Eject the plate from the plate-reader

ii) Start the reaction by adding 10µL of 100mM of INO stock solution (Tube "INO") into the wells, **<u>except in negative controls, that must be free of INO!</u>**

iii) Replace immediately the plate in the plate-reader and measure A_{340} every 1 or 2 minutes for 30-40mn.

5.2.3. Data analysis and calculation

For each well, including for control assays and blanks, plot the A_{340} = f(t) curve as recorded during the incubation phase (reaction). Determine the absorbance rate per minute for every assay (AR_{Assay}) and blank (AR_{Blank})

Calculate $\overline{AR}_{Blank}$, the mean of AR_{Blank} measurements.

Calculate ADK activity for every assay as follows:

$$\text{ADK Activity (in nmol / mn / mL)} = \frac{AR_{Assay} - \overline{AR}_{Blank}}{\varepsilon \,.\, l} \times 10^{6}$$

where: ε is the molar extinction coefficient of $NADH_2$ at 340nm : $\varepsilon = 6220\ M^{-1}.cm^{-1}$
l is the path-length, expressed in cm. l = 0.789 for a 200µL round-bottom well of 96-well microplate (Corning, Costar®, ref. 3797)

Optional: for any positive signals of ADK inhibition, a positive control must be run on IMPDH to check that inhibition does not concern IMPDH enzyme. In this case, the conditions of reaction are the same except that IMPDH is at 0.25mU/mL (final concentration), no ADK enzyme is added and reaction is started with 10µL of 100mM IMP instead of INO.