

## PRECICE® ADK Phosphorylation Assay Kit

**For research use only. Not for use in diagnostic procedures**

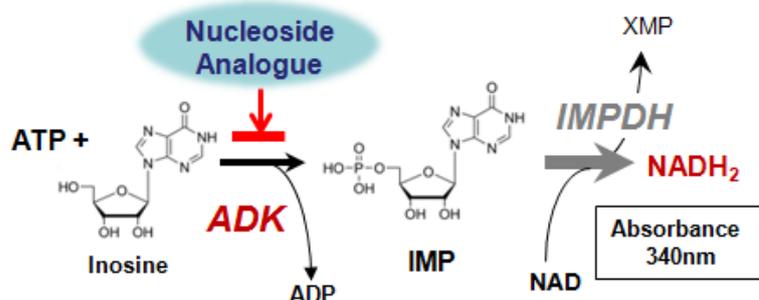
### I. Introduction

**ADK Phosphorylation Assay Kit** was specially designed to evaluate the phosphorylation of novel nucleoside analogues. The assay allows the test of 12 analogues at 7 different concentrations at the same time (or 6 analogues in duplicate).

This assay is based on competitive inhibition of inosine (IR) phosphorylation by ADK enzyme. In the absence of nucleoside competitor, adenosine kinase phosphorylates inosine resulting in the formation of IMP.

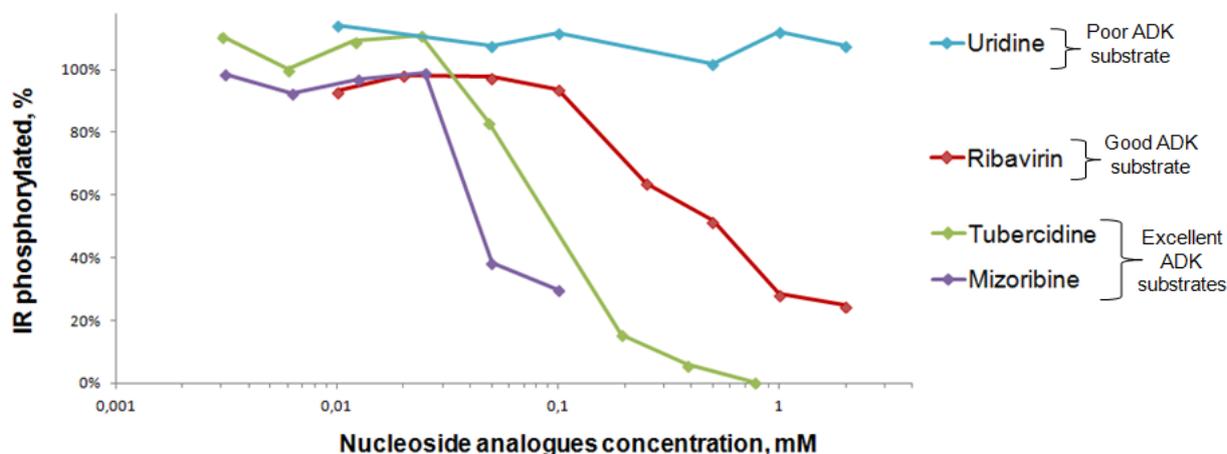
IMP is further oxidized by IMPDH to XMP and NADH<sub>2</sub>. This coupling reaction is immediate when IMPDH activity is much higher than ADK activity in the assay. The enzymatic activity of ADK, which corresponds to the formation kinetics of IMP, is then stoichiometrically and directly monitored by the formation kinetics of NADH<sub>2</sub>.

In the presence of nucleoside competitor, the phosphorylation of inosine, poor ADK substrate, is inhibited detected as a decrease in NADH<sub>2</sub> formation.



This assay was validated with antiviral nucleoside analogues such as mizoribine, tubercidine, ribavirin.

#### Competitive inhibition of inosine phosphorylation by nucleoside analogues



**Figure 1.**

2µL of nucleosides and nucleoside analogues at different concentrations were added to 96-well plate, followed by the addition of 200µL of standard reaction mix containing DTT, NAD, IMPDH and ADK. 15 min later, the reaction was started by the addition of 50mM ATP solution (10µL per well) and the increase in absorbance at 340nm was followed for 30min.

#### **Results:**

Mizoribine, tubercidine and ribavirin competitively inhibit inosine phosphorylation by ADK which totally consistent with previously published data on their substrate properties.

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

## II. Kit Contents

A standard PRECICE<sup>®</sup> ADK Phosphorylation Assay Kit (one 96-well plate) contains:

1. one tube "IMPDH"
2. one tube "Cofactor 1"
3. one tube "Cofactor 2"
4. one tube "Cofactor 3"
5. one vial "Reaction Buffer 5x",
6. one tube "Human ADK enzyme"
7. Transparent 96-well plate (round-bottom 96-well plate Corning, Costar<sup>®</sup>, ref. 3797)

## III. Equipments required

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

## IV. Storage

PRECICE<sup>®</sup> ADK Phosphorylation 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<sup>®</sup> 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 Protocol I - for nucleoside analogues ADK competitors

**Important:** Spin all tubes before solubilizing.

### V.1. Preparation of Nucleoside analogues dilutions

Prepare 7 different dilutions for each of the nucleoside analogue to be tested.

Dispose 2µL of each dilution per well (use one column of the plate for one nucleoside analogue, the 8<sup>th</sup> well is used as a control with no analogue).

### V.2. Reconstitute IMPDH enzyme

Add 250µL of deionized water to "IMPDH" tube. Agitate gently until complete dissolution of the powder.

### V.3. Preparation of standard reaction buffer (1x)

i) Add the content of "Reaction Buffer 5x" tube to 16ml of deionized water to prepare "Reaction Buffer 1x".

ii) Transfer quantitatively the content of 2 tubes with "Cofactor 1", "Cofactor 2" to the tube with "Reaction buffer 1x".

**Important: Do not add "Cofactor 3"!**

To do so:

iii) - pipet 1ml of "Reaction buffer 1x" to each tube with cofactors and mix them by inverting or pipeting up and down until the powder dissolved.

iv) - transfer by pipeting the content of two tubes back into a vial "Reaction buffer 1x";

v) - 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.

vi) Transfer quantitatively the content of "IMPDH" tube to "Reaction buffer 1x" with co-factors.

vii) 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".

*Composition of complete reaction buffer: 100mM Tris-HCl, 100mM KCl, 12mM MgCl<sub>2</sub>, BSA 1mg/ml, 5mM DTT, 5mM NAD, 0,1mM inosine, IMPDH 30mU/ml; human recombinant ADK 6mU/ml.*

### V.4. Preparation of ATP solution for starting the reaction

Add 1ml of deionized water to the tube "Cofactor 3" containing ATP powder. Mix until dissolved. 50mM ATP solution is obtained.



## VI. Following ADK activity in vitro

### VI.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. Start the reaction and incubate (30')

- 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 30-40min.
- iii) Start the reaction by adding 10µL of 50mM ATP per well (2,5mM ATP final concentration).
- iv) Place the plate in the plate-reader and start the measurements. Record second set of data.