

## PRECICE® Deoxycytidine Kinase (dCK) Phosphorylation Assay Kit

For research use only. Not for use in diagnostic procedures

### I. Background

**Human deoxycytidine kinase (dCK)** is a central enzyme in the salvage pathway of deoxynucleotide synthesis. This pathway is essential for supplying resting cells with dNTPs for DNA repair; maintaining mitochondrial DNA synthesis and activating many therapeutic nucleoside analogues. Human deoxycytidine kinase (dCK) has unusually broad specificity. It phosphorylates purine deoxynucleosides (dAR, dGR); pyrimidine deoxynucleosides (dCR); ribonucleosides (CR) and L-nucleosides (non-physiological) as 3TC (lamivudine). It can use ATP or UTP as phosphate donors, with UTP preferred.

dCK is the activation enzyme for many anticancer and antiviral nucleoside analogues: Cytarabine; Gemcitabine; Cladribine; Fludarabine and L-nucleosides like lamivudine (3TC). Structural studies have shown how dCK accommodates both D- and L-nucleosides, explaining why it activates drugs of both chiralities.

### II. Principle

**PRECICE® dCK Phosphorylation Assay Kit** is specifically designed to evaluate the phosphorylation of novel ribonucleoside and deoxyribonucleoside analogues by human recombinant deoxycytidine kinase (dCK) provided with the kit.

NOVOCIB's human dCK enzyme is an active, purified 33 kDa protein obtained by RT-PCR amplification of mRNA extracted from human hepatoma cells, expressed in *E. coli*, and characterized for its substrate properties (Km and Vmax). Its kinetic parameters were compared with published data for several nucleoside analogues (Table 1).

**Table 1. Characterization of the substrate properties (Km and Vmax) of nucleoside analogues for NOVOCIB human deoxycytidine kinase, compared with published kinetic parameters for well-characterized nucleoside analogues such as aracytidine, gemcitabine, cladribine, and lamivudine.**

Substrate	Km (µM) Novocib dCK	Vmax (µmol/mg/min) Novocib dCK	Km (µM) Published	Vmax (µmol/mg/min) Published	Reference
Deoxycytidine	0.577	0.026	0.16	0.033	Johansson & Karlsson, 1995 <sup>1</sup>
			1.3	0.069	Usova & Eriksson, 1997 <sup>2</sup>
			0.57	0.004	Someya et al., 2003 <sup>3</sup>
Gemcitabine	42.71	0.325	—	—	—
Deoxyadenosine	150.5	1.08	115	—	Sabini et al., 2008 <sup>4</sup>
			480	1.5	Johansson & Karlsson, 1995 <sup>1</sup>
Aracytidine	6.81	0.224	15	0.009	Someya et al., 2003 <sup>3</sup>
Cladribine	56.5	0.285	89	0.126	Usova & Eriksson, 1997 <sup>2</sup>
			24	0.76	Johansson & Karlsson, 1995 <sup>1</sup>

**PRECICE® dCK Phosphorylation Assay Kit** is based on the competitive inhibition of deoxyinosine (dIR) phosphorylation by human deoxycytidine kinase (dCK) in the presence of a nucleoside analogue. The assay enables the simultaneous testing of 12 analogues at 7 concentrations, or 6 analogues in duplicate.

The reaction sequence is as follows:

- In the absence of a nucleoside competitor, dCK phosphorylates deoxyinosine, producing dIMP.
- dIMP is immediately oxidized by IMPDH to dXMP, with concomitant formation of NADH<sub>2</sub>.
- In the presence of a nucleoside competitor, phosphorylation of deoxyinosine (a poor dCK substrate) is inhibited, resulting in a decrease in NADH<sub>2</sub> formation.

Because IMPDH activity is supplied in large excess, the coupling reaction proceeds instantly, ensuring that the rate-limiting step is dCK activity. Thus, the enzymatic activity of dCK—corresponding to the formation rate of dIMP—is stoichiometrically and directly monitored by measuring NADH<sub>2</sub> production at 340 nm (Fig. 1).

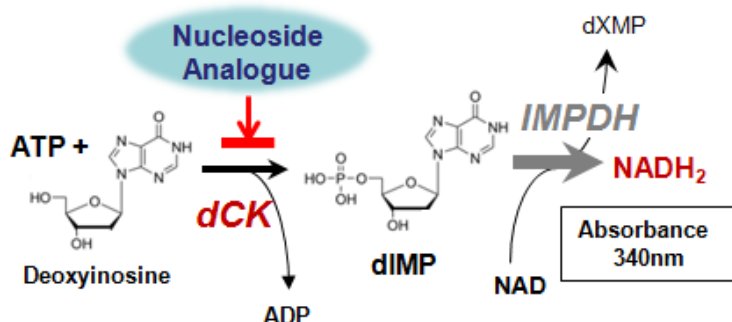
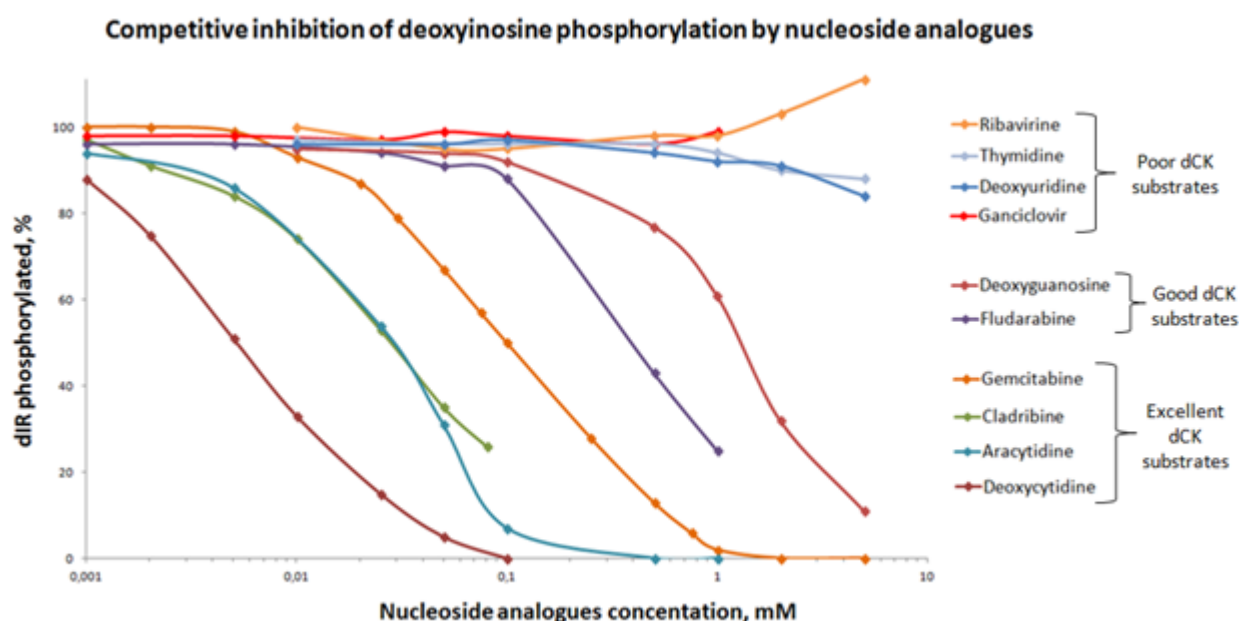


Figure 1. Enzymatic principle of PRECICE® dCK Phosphorylation Assay Kit.

**PRECICE® dCK Phosphorylation Assay** was validated using anticancer nucleoside analogues such as gemcitabine, cladribine, and aracytidine—compounds known to be efficiently phosphorylated by tumor cells—as well as natural pyrimidine and purine nucleosides. Deoxycytidine, cladribine, aracytidine, and gemcitabine all **competitively inhibit** deoxyinosine phosphorylation by dCK



### III. Equipment 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 dCK enzyme *in vitro*, in a range allowing this measurement by spectrophotometry as described here below. NovoCIB does not guarantee the use of its PRECICE® dCK 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.

#### IV. Kit Content:

A standard PRECICE® dCK Phosphorylation Assay Kit (one 96-well plate) contains:

1. one tube "Cofactor 1";
2. one tube "Cofactor 2";
3. one tube "Cofactor 3";
4. one tube "Deoxyinosine";
5. one tube "Highly active IMPDH" (lyophilized);
6. one tube "Human dCK enzyme", (lyophilized);
7. one tube "Gemcitabine", (lyophilized);
8. one tube "Reaction Buffer 5x", 4ml (provided in 50ml tube)
9. Transparent 96-well plate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

#### V. Preparation of "Reaction buffer"

**Important: Spin the tubes before opening to recover their content in the bottom.**

1. Add 16ml (corresponding to 16g) of deionized water to provided 50-mL tube with "Reaction Buffer 5x" to prepare "Reaction Buffer 1x";
2. Add 1ml "Reaction Buffer 1x" to five following tubes:  
" Cofactor 1", "Cofactor 2", "Highly active IMPDH"; "Human dCK enzyme"; "Deoxyinosine".  
Close, leave for 5 min, agitate or vortex until complete solubilization of powder, transfer the content tube back into a vial "Reaction buffer 1x". **Important: Do not add "Cofactor 3" (ATP)!**
3. Add 1ml of deionized water to the tube "Cofactor 3" containing 30mg of ATP powder. Mix until dissolved. 50mM ATP solution is obtained.

**Once prepared, complete "Reaction mixture 1x" containing cofactors and enzymes should be used on the same day.**

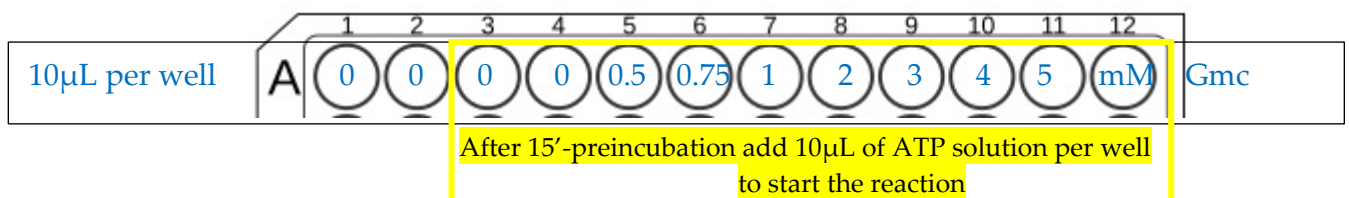
#### VI. Preparation of nucleoside analogues

1. Add 100µL of dH2O to "Gemcitabine" tube to prepare 25mM solution;

Prepare different dilutions for each of the nucleoside analogue to be tested. Add 10µL per well.

##### Example of gemcitabine dilutions

Gemcitabine, stock concentration	0mM	0.5mM	0.75mM	1mM	1.5mM	2mM	3mM	4mM	5mM
Gemcitabine 25mM, µL	0	2	3	4	6	8	12	16	20
H2O, µL	100	98	97	96	94	92	88	84	80
Gemcitabine, final concentration in well, µM (10µL per well)	0	25µM	37.5µM	50µM	75µM	100µM	150µM	200µM	250µM

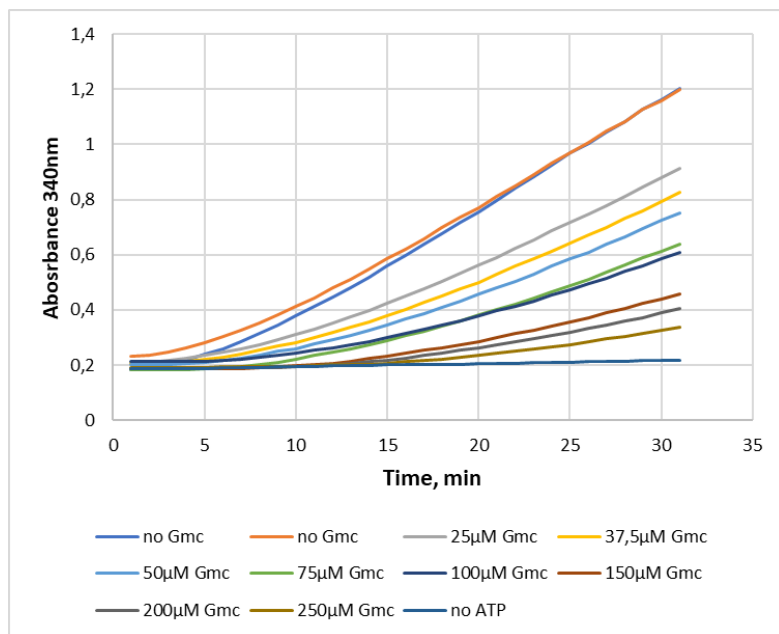


#### VII. Microplate preparation

1. **Positive control.** Add 10µL of each gemcitabine solutions per well (prepare as in Section VI);
2. Add 10µL of studied nucleoside analogue solution per well;
3. Add 200µL of complete "Reaction mixture 1x" containing NAD, DTT, IMPDH, dCK and deoxyinosine per well;
4. Program plate reader for kinetics absorbance reading (every 1 min), 37°C.

5. **Preincubation 15'**: Insert the plate into the reader pre-heated at 37°C, agitate for 1min and preincubate the reaction at 37°C for 15min.
6. To start the reaction, add 10µL of 50mM ATP solution per well;
7. Insert the plate into the reader pre-heated at 37°C, agitate for 1min and monitor the reaction at 340nm at 37°C for 30min with data collection every min.

Typical results obtained with gemcitabine are shown on Table2 and Figure 2 (batch 280226).



**Figure 2. Effect of gemcitabine on deoxyinosine phosphorylation by human deoxycytidine kinase (dCK)**

Time course of dIMP formation by human recombinant dCK incubated in the absence and presence of increasing concentration of gemcitabine. After vigorous shaking, the absorbance at 340nm was monitored at 37°C using iEMS microplate reader (Thermo) and round-bottom 96-well microplate (Corning® ref 3797).

**Table 2.**

Time, min	Absorbance 340 nm											
	no ATP no Gmc	no ATP no Gmc	no Gmc	no Gmc	25µM Gmc	37,5µM Gmc	50µM Gmc	75µM Gmc	100µM Gmc	150µM Gmc	200µM Gmc	250µM Gmc
0	0,187	0,188	0,19	0,19	0,187	0,178	0,178	0,184	0,184	0,182	0,183	0,182
1	0,187	0,188	0,204	0,205	0,189	0,178	0,179	0,184	0,184	0,183	0,183	0,181
2	0,187	0,188	0,227	0,228	0,195	0,18	0,178	0,184	0,184	0,182	0,183	0,181
3	0,187	0,188	0,258	0,259	0,207	0,187	0,179	0,185	0,185	0,182	0,182	0,181
4	0,189	0,188	0,294	0,295	0,223	0,197	0,18	0,186	0,186	0,183	0,183	0,181
5	0,189	0,188	0,335	0,336	0,243	0,212	0,181	0,19	0,188	0,184	0,184	0,181
6	0,189	0,187	0,38	0,382	0,266	0,229	0,182	0,196	0,191	0,184	0,184	0,182
7	0,189	0,187	0,428	0,431	0,292	0,249	0,185	0,205	0,197	0,186	0,185	0,182
8	0,189	0,187	0,479	0,483	0,321	0,271	0,191	0,216	0,205	0,188	0,186	0,183
9	0,19	0,187	0,533	0,537	0,353	0,296	0,198	0,23	0,216	0,192	0,188	0,184
10	0,19	0,188	0,588	0,594	0,386	0,323	0,208	0,245	0,228	0,197	0,189	0,185
11	0,191	0,187	0,645	0,651	0,422	0,352	0,219	0,262	0,242	0,204	0,192	0,186
12	0,191	0,188	0,702	0,711	0,459	0,383	0,231	0,28	0,257	0,212	0,195	0,187
13	0,193	0,188	0,761	0,771	0,498	0,414	0,244	0,3	0,274	0,223	0,201	0,19
14	0,193	0,188	0,819	0,831	0,537	0,447	0,26	0,321	0,292	0,234	0,206	0,192
15	0,194	0,188	0,878	0,893	0,579	0,483	0,278	0,343	0,311	0,245	0,213	0,194
16	0,194	0,188	0,938	0,954	0,621	0,517	0,293	0,366	0,331	0,259	0,221	0,198
17	0,194	0,189	0,997	1,016	0,664	0,554	0,311	0,39	0,352	0,273	0,23	0,202
18	0,195	0,189	1,055	1,077	0,709	0,593	0,332	0,417	0,376	0,289	0,241	0,209
19	0,195	0,189	1,114	1,138	0,753	0,63	0,351	0,442	0,398	0,304	0,251	0,215
20	0,195	0,189	1,171	1,198	0,798	0,669	0,371	0,469	0,422	0,321	0,264	0,222
21	0,195	0,19	1,229	1,258	0,843	0,708	0,393	0,496	0,447	0,338	0,277	0,23
22	0,196	0,19	1,286	1,318	0,889	0,748	0,415	0,523	0,47	0,354	0,288	0,238
23	0,196	0,191	1,342	1,377	0,936	0,79	0,439	0,554	0,498	0,375	0,303	0,248
24	0,196	0,191	1,396	1,435	0,98	0,828	0,46	0,581	0,522	0,393	0,316	0,257
25	0,196	0,191	1,45	1,491	1,027	0,869	0,484	0,611	0,549	0,412	0,331	0,268
26	0,196	0,192	1,502	1,547	1,072	0,91	0,508	0,64	0,575	0,431	0,346	0,278
27	0,197	0,192	1,555	1,601	1,118	0,95	0,532	0,67	0,602	0,452	0,361	0,29
28	0,197	0,192	1,606	1,655	1,163	0,99	0,556	0,698	0,628	0,471	0,376	0,301
29	0,198	0,193	1,656	1,708	1,209	1,031	0,582	0,731	0,658	0,493	0,393	0,313
30	0,198	0,193	1,706	1,76	1,254	1,071	0,607	0,761	0,686	0,514	0,41	0,325
	no ATP no Gmc	no ATP no Gmc	no Gmc	no Gmc	25µM Gmc	37,5µM Gmc	50µM Gmc	75µM Gmc	100µM Gmc	150µM Gmc	200µM Gmc	250µM Gmc
Rate, AU/min	0,000	0,000	0,054	0,056	0,038	0,032	0,015	0,020	0,018	0,011	0,007	0,004
dIMP formation, % of control			98%	102%	69%	58%	27%	37%	32%	21%	14%	8%