Purine and pyrimidine nucleotides play crucial roles in major cell functions: they are the basic building blocks of nucleic acids, are the components of key cell metabolic processes such as energy metabolism, or the biosynthesis of phospholipids and glycoproteins and are involved in cell signaling mechanisms.

Nucleosides and nucleotides are therefore of great interest in cell biology research, for many application fields in pharmacology and drug discovery, as well as in foodstuffs QC.

**Nucleosides Metabolism Explorer**

NOVOCIB is a French biotechnology company which was awarded with three prestigious national prizes* and has specialized and developed a strong expertise in the study of nucleotides metabolism and the analysis of nucleotides and nucleosides.

Through PRECICE®, a unique platform specially dedicated to the exploration of nucleotide metabolism, the production of recombinant enzymes and the development of innovative enzymatic assays, NOVOCIB provides a wide range of products and services.

**Human Recombinant Enzymes**

- **Screening assays**
- **Whole Cell assays**
  - IMPDH II, PNP
  - Nucleoside kinases – AK, dCK, YMPK, cN-II...
  - Coupled Phosphorylation assays: dCK-YMPK
  - Coupled Nucleoside kinase – IMPDH assay

**Nucleosides Profiling Analytical services: an Essential Tool for Mode of Action Study**

- Extraction, identification & quantification
- Quantification of ribo-, deoxyribonucleotides and nucleotides co-factors in treated cells
- Analysis of drug's mode of action
- Comparison with nucleotide profiles of reference drugs
- Specificity of drug’s effects
- Other customized services for nucleotide analysis

**Other assays for Food QC**

- Fish Freshness measurement (K-value)
- Nucleotide analysis in Baby Milk

* Double laureate of the French National Contests for Innovative Companies (French Ministry of Research), "Emergence” category in 2003, "Creation & Development” category in 2005, Laureate of the French Senate "Tremplin Entreprises” contest in 2004. In 2009, NovoCIB won the "Talent de l'Innovation", in research category. Dr Balakireva, CEO & Founder, was named “Femme décideur (women and decision-makers) 2009” (Jury Prize)
Enzymes, Assays & Kits

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Other services are continuously under development at NOVO CIB.

Please, do not hesitate to contact us

Some of our non-confidential Client References *
- The University of Chicago, IL, USA
- The University of Texas Medical Branch, TX, USA
- INSERM Grenoble and INSERM Créteil, France

... and Project Partners *
- CNRS, Lyon, France
- Université Claude Bernard, Lyon, France
- Université de Clermont-Ferrand, France

* Most of the services provided by NovoCIB to its clients – biotechnology and pharmaceutical companies, public research organizations – are contracted under secrecy agreement.
IMPDH II, a choice target for major therapeutic applications

Catalytic activity

Inosine Monophosphate Dehydrogenase (IMPDH) converts inosine 5’-monophosphate (IMP) to xanthosine 5’- monophosphate (XMP) using NAD⁺ as a cofactor.

The oxidation of IMP to XMP is considered as the pivotal step in the biosynthesis of guanine nucleotide, whose pool controls cell proliferation and many other major cellular processes. The decrease in guanine nucleotide resulting from IMPDH inhibition interrupts the nucleic acid synthesis in proliferating cells. The involvement of IMPDH in de novo guanine nucleotide biosynthesis makes IMPDH a crucial enzyme in cell proliferation and differentiation. IMPDH is recognized as a validated target for several major therapeutic areas. IMPDH inhibitors are exploited as antiviral (e.g. ribavirine), antiparasitic, antimicrobial, antileukemic and immunosuppressive agents. IMPDH Type II is the predominant isoenzyme of the enzyme and is selectively expressed in proliferating cells, including lymphocytes and tumor cells.

IMPDH in immunology

IMPDH is highly active in lymphocytes. It is a validated target to treat immunological diseases and to induce immunosuppression (CellCept®, a mycophenolic acid (MPA) prodrug - Roche – CHF1.85 Bn as an immunosuppressive agent in 2006, orphan drug designation in 2006 for Myasthenia Gravis, Phase III in Lupus Nephritis). IMPDH is also recognized as an excellent target for the treatment of psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

IMPDH in oncology

IMPDH, and particularly Type II, which is overexpressed in tumor cells, is considered as a highly potent target for cancer chemotherapy. Several IMPDH inhibitors are under development for the treatment of Acute and Chronic Myelogenous Leukemia (AML, CML) and other cancers (pancreas, colon, bladder...). Additionally, it has been shown that the use of IMPDH inhibitors counteracts the drug resistance that may appear in certain tumors. For instance, methotrexate resistance is directly related to the overexpression of IMPDH, whose inhibition restores the drug efficacy. Combination with other anti-cancer drugs extends the potential application of IMPDH inhibitors.

Current development of IMPDH inhibitors

CellCept®, ribavirin, mizoribine and tiazofurine are examples of currently used drugs that target IMPDH. Benzamide riboside, tiazofurine, MPA are under development in Phase II/III in leukemia: results are judged very encouraging. The IMPDH II atomic structure has been resolved and it provides a valuable background for further leads optimization. Besides nucleosides analogues, NCEs have been identified as IMPDH inhibitors and enter development trials (e.g. AVN-944: Phase I in advanced hematologic malignancies, Phase II in pancreatic and other solid tumors).

All this demonstrates how promising the research of new IMPDH inhibitors is and why the inhibiting activity of compounds is worth being evaluated on such a highly pertinent target.

5. B. J. Barnes et al. (2001): Induction of Tmolt4 Leukemia Cell Death by 3,3-Disubstituted-6,6-pentamethylene-1,5-diazabicyclo[3.1.0]hexane-2,4-diones: Specificity for Type II Inosine 5’-Monophosphate Dehydrogenase J. Pharm. Exp. Therap. 298(2), 790-796
Inosine Monophosphate Dehydrogenase Type II

Human, recombinant expressed in E. coli

E.C. 1.1.1.205

Synonyms: IMP Dehydrogenase Type II, IMPDH II

Description

NOVOCIB’s IMPDH II is a human recombinant Inosine Monophosphate Dehydrogenase Type II expressed in E. coli. It has an apparent molecular weight of ca. 56 kDa.

Inosine monophosphate dehydrogenase converts inosine 5’-monophosphate to xanthine 5’-monophosphate using NAD as a cofactor.

IMPDH is involved in de novo guanine nucleotide biosynthesis. It plays a major role in cell growth and in the malignancy of some tumors. Additionally, guanine nucleotide is needed for lymphocyte proliferation.

IMPDH II is the predominant isoform of IMPDH. It is recognized as a validated target to treat a wide range of cancers and infectious diseases and to prevent lymphocytes proliferation (for further details, see “IMPDH II, a choice target for major therapeutic applications”).

Storage: –70 °C in a solution containing 50 mM KH₂PO₄, pH 8.0, 1 mM EDTA, 0.1 mM DTT, 50% glycerol.

Unit Definition: One unit of IMPDH Type II catalyzes the formation of 1 µmole of NADH per minute at pH 8.0 at 25 °C

Specific Activity: \( \frac{1}{0.035 \text{ unit/mg protein}} \)

Purity controlled by SDS-PAGE

Assay condition:

KH₂PO₄ 0.1M, pH 7.8, NAD 180µM, DTT 1mM, 0.13mU of human recombinant IMPDH II (2µl at 0.081 U/mg protein)

Incubation at 25°C. Reaction started by adding IMP at various concentrations. NADH formation was measured in an iEMS Reader MF (Labsystems, Finland) microtiter plate reader at 340nm.

At 25°C, \( V_{\text{Max}} = 0.8 \mu \text{M.mn}^{-1} \), \( K_M = 124.4 \mu \text{M} \)

**IMPDH inhibition assays**

**In vitro Assay**

for Screening & Kinetic Analysis (IC₅₀)

- with Human Recombinant IMPDH II
- with Bacterial (Staphylococcus aureus) IMPDH

**Whole Cell Assay**

for Screening & Kinetic Analysis (IC₅₀)
in Whole Cell system

**Applications:**

- Chemical library screening,
- Hit selection, Lead optimization
- Complementary studies for drug development
**IMPDH II - In vitro Assay**

**Aim:** To screen compounds for their abilities to inhibit human IMPDH II *in vitro.*

To determine the inhibition kinetics of a given compound on human recombinant IMPDH II and measure its IC\textsubscript{50} value.

**Human IMPDH II:** The IMPDH II enzyme used in the assays is a human recombinant IMPDH II, cloned by NovoCIB from human cells, expressed in *E. coli,* and produced and purified in NovoCIB's laboratory (see sheet # E-Nov 1 for further information).

**Enzyme QC:** The IMPDH II enzyme purity is controlled before every assay by SDS-PAGE. A standard operating procedure (SOP) is followed to measure IMPDH enzymatic activity.

**Enzyme concentration:** Bradford method

**Enzyme specific activity:** 35 mU/mg protein - 1 unit of IMPDH Type II catalyzing the formation of 1 µmole of NADH per minute at pH 8.0 at 25 °C

**Replicate assays:** One point is defined as a well per compound and per concentration tested. IMPDH *in vitro* Assays are usually performed in duplicate (2 wells per compound and per concentration). Triplicates are available upon request.

**IMPDH II inhibition control:** Mycophenolic Acid (MPA), dissolved in DMSO, is used as positive control for IMPDH II inhibition. Other positive control than MPA can be used if available. Both negative and positive controls are done in duplicate.

**Enzymatic Reaction:** The assays are performed at 25°C or 37°C in 200µl of reaction buffer on 96-well microplate.

**Reaction buffer is:** KH\textsubscript{2}PO\textsubscript{4} 0.1M, pH7.8, NAD 180µM, DTT 1mM

**Plotting:**

\[ \text{NADH formation} = \text{fractional activity} = \frac{'A'}{A_{\text{max}}} = -\frac{\epsilon_{\text{NADH}} \cdot \text{light pass in a well}}{\epsilon_{\text{NADH}} \cdot \text{light pass in a well}} \]

**IMPORTANT:** Client-specified alterations can be accommodated.

**Automation:** Pipetting is done by a Multiprobe\textsuperscript{®} II Robotic Liquid Handling System (Packard BioScience).

**Procedure:** Every assay, from one to 90 points, is done with one negative control, containing DMSO with no inhibitor, and:

- For Screening Assays: 2 positive controls containing MPA as an IMPDH inhibitor at final concentrations of 50nM and 50µM
- For Kinetics Analysis (IC\textsubscript{50}): 11 positive controls containing MPA as an IMPDH inhibitor, at 11 concentrations which are equally spaced by 3-fold dilutions to cover a 4.8-log wide range, as follows:

<table>
<thead>
<tr>
<th>MPA (nM)</th>
<th>0.17</th>
<th>0.51</th>
<th>1.52</th>
<th>4.56</th>
<th>13.69</th>
<th>41.07</th>
<th>123.2</th>
<th>370</th>
<th>1109</th>
<th>3326</th>
<th>9980</th>
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</thead>
<tbody>
<tr>
<td>log\textsubscript{10}</td>
<td>-0.77</td>
<td>-0.29</td>
<td>0.18</td>
<td>0.66</td>
<td>1.14</td>
<td>1.61</td>
<td>2.09</td>
<td>2.57</td>
<td>3.04</td>
<td>3.52</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Controls are done in duplicate. If an additional microplate is needed, it includes the complete set of controls (in duplicate). Additional concentrations of inhibitor can be tested.

**NADH formation** is measured every mn for 30 mn in an iEMS Reader MF (Labsystems, Finland) microtiter plate reader at 340nm. Activity is determined by: \[ \Delta A / \text{Abs} \text{max} \text{.} \text{t} \] where \( \text{Abs} \text{max} \text{.} \text{t} \) is the molar extinction coefficient for NADH at 340nm \((= 6220 \text{M}^{-1} \text{cm}^{-1})\), \( \Delta A \) is the absorbance variation at 340nm from \( t = 0 \) to \( t \), \( p \) is the light pass in a well \((= 0.825 \text{ cm} \text{ for} 200\upmu \text{l} \text{well})\), \( t \) is the maximal time \((\leq 30\text{mn})\) at which velocity (NADH formation rate) remains constant.

*(Optional)* For every positive result of a Screening assay, a confirmation by HPLC (Agilent 1100 series) of IMPDH II inhibition can be performed upon request by measuring IMP, XMP, NAD\textsuperscript{+} and NADH concentrations in the assay and by comparison with negative and positive controls.

**For Kinetics Analysis,** IC\textsubscript{50} is determined by plotting the fractional activity - ratio between the maximal activity observed *(i.e. without inhibitor)* and the activity at each compound concentration – as a function of inhibitor concentration. IC\textsubscript{50} is then calculated using a standard four-parameter nonlinear regression analysis.

**Plotting:** As far as possible, the inhibitor concentration range is determined in order to get *:

- half of the data points +/- 1above the IC\textsubscript{50} value or half +/- 1 below
- well-defined top and bottom plateau values, at least within a 15% margin of theoretical values.

* Abiding by these constraints depends on the availability of information about the compound before starting the assay. When the results of the assay do not meet two of these three constraints, whereas IMPDH II inhibition by the compound is demonstrated, an additional assay can be performed with ad hoc alterations of the procedure *(e.g. inhibitor concentration range, additional points, substrate concentration...)*.

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Bacterial IMPDH (Staphylococcus aureus)  
Recombinant, expressed in E.coli  
EC 1.1.1.205

Description

NOVOCIB’s bacterial IMPDH is a recombinant protein of ca. 53kDa cloned by PCR amplification of guaB gene of Staphylococcus aureus and expressed in E.coli.

Today, antibiotic resistance is one of the world’s most important public health problems. There is an urgent need for new antibiotic compounds acting on new targets. One attractive strategy for developing new antibiotics consists in inhibiting bacterial IMPDH, an enzyme involved in the de novo synthesis of purine nucleotides, and therefore, necessary for bacterial cell growth and division.

Mammalian and bacterial IMPDHs are known to have significantly different kinetic properties and inhibitor sensitivities (1, 2). The experiments done with previously cloned human IMPDH 2 (ref. # E-Nov 1) and bacterial IMPDH of Staphylococcus aureus, are illustrated below. In agreement with published data, mycophenolic acid (MPA) inhibits human IMPDH type II >20-times more efficiently than bacterial IMPDH with IC_{50} values of 100nM and 2.6µM, respectively (A). In contrast, mizoribine monophosphate displays the opposite selectivity (B). It is a more potent inhibitor of bacterial IMPDH with respective IC_{50} values of 12nM and 185nM for bacterial and human enzymes.

Both bacterial recombinant IMPDH and human recombinant IMPDH are available from NOVOCIB providing the tools for selection of species-specific IMPDH inhibitors.

Unit Definition:

One unit of IMPDH converts 1.0 µmole of IMP and NAD to XMP and NADH per minute at pH 8 at 37°C.

Specific Activity:

≥ 0.3 unit/mg protein.

Purity:

controlled by 12%AA SDS-PAGE.

IMP DH inhibition:

Effect of MPA (A) and mizoribine monophosphate (B) on human recombinant IMPDH II (red curve) and bacterial recombinant IMPDH of Staphylococcus aureus. (blue curve) Enzymatic assays performed in duplicate are carried out at 37°C in 0.1M KH2PO4 buffer pH 8.0 in the presence of 1mM DTT, 200µM NAD, 200µM IMP, 60nM IMPDH II or 95nM IMPDH S.aureus. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm.

Monophosphorylated mizoribine is produced by enzymatic phosphorylation of mizoribine (MPBiochemicals) by adenosine kinase (Novocib E-Nov5).

Related products:

- Coupled nucleoside kinase – IMPDH assay
- Human recombinant IMPDH II
- IMPDH II inhibition in vitro assay
- Human Adenosine kinase (AK)

References:

Catalytic activity
Purine Nucleoside Phosphorylase (PNP) is involved in salvage pathway of the purine metabolism. PNP catalyzes the cleavage of the glycosidic bond of ribo- or deoxyribonucleosides, in the presence of inorganic phosphate as a second substrate, to generate the purine base and ribose- or deoxyribose-1-phosphate. The reaction is reversible for natural substrates:

\[
\text{Purine nucleoside} + \text{Pi} \rightleftharpoons \text{Purine base} + \text{(deoxy)ribose-1P}
\]

Therapeutic potential of PNP inhibitors
PNP deficiency leads to T-lymphocytopenia, usually with no apparent effects on B-lymphocyte function. These symptoms suggest possible chemotherapeutic applications of potent inhibitors of PNP, as selective immunosuppressive agents, to treat T-cell leukemias or T-cell-mediated autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis.\(^1\) The decrease in plasma and urine levels of urate is an additional symptom of PNP deficiency. PNP inhibitors may contribute to treat hyperuricemic states, such as secondary or xanthine gout.

Some PNP inhibitors have undergone clinical trials for the treatment of cutaneous T-cell lymphoma, acute lymphoblastic leukemia (ALL), HIV infections, and psoriasis.

Peldesine (BCX-34) was granted orphan drug status for the treatment of T-cell lymphoma and reached phase III as an immunomodulator for inflammatory autoimmune diseases.

Forodesine (BCX-1777) has US orphan drug status for the treatment of non-Hodgkin's lymphoma, cutaneous T-cell lymphoma (CTCL), chronic lymphocytic leukemia (CLL) and related leukemias, including prolymphocytic leukemia (PLL), adult T-cell leukemia, hairy cell leukemia and acute lymphocytic leukemia (ALL).\(^2\) In December 2006, Forodesine was designed Orphan drug in Europe for ALL.\(^3\)

PNP inhibitors are also investigated to prevent the cleavage, and the resulting deactivation of Nucleoside Analogues by PNP.

Note: Protozoan parasites are auxotrophic for purine and have their own PNPs which have specific activities and properties that differ from the human PNP. Protozoan parasites PNPs are considered to be reasonable target against infection (e.g. Plasmodium falciparum).\(^4\)

PNP, a threat for therapeutic efficacy of Nucleoside Analogues
In vivo, phosphorylation is highly favoured over purine nucleoside synthesis and is coupled with two additional enzymatic reactions: oxidation of the liberated purine base by xanthine oxidase (XO) and its phosphoribosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT).\(^5\) Thus, PNP plays a key role in the salvage pathway of the purine metabolism, enabling the cell to utilize purine bases recovered from metabolized purine ribo- and deoxyribonucleosides to synthesize purine nucleotides.

This phosphorylation reaction of purine nucleosides by PNP has a direct impact on the therapeutic efficacy of Nucleoside Analogues. Antitumour or antiviral nucleoside analogues are likely to be cleaved by PNP before being phosphorylated by the cell nucleoside kinases and converted to the active nucleotide form. For instance, 2',3'-dideoxyguanosine (ddG), 9-β-D-ribobutynosyl guanine (AraG)\(^6\) as well as one of its produg, Nelarabine (Arranon, GSK),\(^7\) which is intracellularly converted to AraG by Adenosine deaminase (ADA), are PNP resistant nucleoside analogues, whereas 2',3'-dideoxyinosine (ddl)\(^8\) is easily cleaved in vivo by PNP.

Since acyclicnucleoside analogues are particularly resistant to cleavage by PNP though phosphorylated by viral thymidine kinases (TK), they are generally considered as excellent candidates as antiviral agents (e.g. aciclovir, ganciclovir).\(^9\)

Note that Ganciclovir is not only PNP resistant, but is also a PNP inhibitor.

PNP, a tool for enzymatic synthesis of Nucleoside Analogues
PNP can be exploited for the reversible reaction that it catalyzes to synthesize nucleoside analogues, for instance with potential antiviral and antineoplastic activities, especially when chemical synthesis is difficult to prepare and / or gives low yields.

(coming soon, “Transribosylation by PNP”)

8. L. C. Gradov et al. (1993): Efficacy and toxicity of 9-β-D-arabinofuranosylguanine (araG) as an agent to purge malignant T-cells from murine bone marrow: application to an in vivo T-cell leukemia model. Leukemia 7(8), 1261-1267
PNP - Purine Nucleoside Phosphorylase

Human, recombinant expressed in E. coli
E.C. 2.4.2.1

Description

Metabolic function
Purine Nucleoside Phosphorylase (PNP) is involved in salvage pathway of the purine metabolism.

Catalytic activity
PNP catalyzes the cleavage of the glycosidic bond of ribo- or deoxyribonucleosides, in the presence of inorganic phosphate as a second substrate, to generate the purine base and ribose- or deoxyribose-1-phosphate. The reaction is reversible for natural substrates:

\[
\text{Purine nucleoside} + \text{Pi} \rightleftharpoons \text{Purine base} + \text{(deoxy)ribose-1P}
\]

NOVO CIB's PNP is a human recombinant Purine Nucleoside Phosphorylase expressed in E. coli. It has an apparent molecular weight of 32.12 kDa.

Interests

PNP inhibition
Several PNP inhibitors have been developed to treat cancer, viral infection and auto-immune diseases.

PNP, a threat for therapeutic efficacy of Nucleoside Analogues
PNP's activity in vivo can be responsible for the cleavage and the subsequent deactivation of Nucleoside Analogues, thus unable to be phosphorylated by nucleoside kinases. The resistance to cleavage by PNP is worth being investigated to increase the therapeutic efficacy of Nucleoside Analogues.

PNP, a tool for enzymatic synthesis of Nucleoside Analogues
PNP can be exploited for the reversible reaction that it catalyzes to synthesize nucleoside analogues, for instance with potential antiviral and antineoplastic activities, especially when chemical synthesis is difficult to prepare and / or gives low yields.

PNP Services

• PNP Inhibition - In Vitro Screening Assay
• PNP Cleavage activity - Nucleoside Resistance Assay
• (coming soon) Transribosylation by PNP
**Aim:** To screen compounds for their abilities to inhibit human PNP *in vitro*.

**Therapeutic potential of PNP inhibitors**

PNP deficiency leads to T-lymphocytopenia, usually with no apparent effects on B-lymphocyte function. These symptoms suggest possible chemotherapeutic applications of potent inhibitors of PNP, as selective immunosuppressive agents, to treat T-cell leukemias or T-cell-mediated autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis.

The decrease in plasma and urine levels of urate is an additional symptom of PNP deficiency. PNP inhibitors may contribute to treat hyperuricemic states, such as secondary or xanthine gout.

Some PNP inhibitors have undergone clinical trials for the treatment of cutaneous T-cell lymphoma, acute lymphoblastic leukemia (ALL), HIV infections, and psoriasis. Peldesine (BCX-34) was granted orphan drug status for the treatment of T-cell lymphoma and reached phase III as an immunomodulator for inflammatory autoimmune diseases. Forodesine (BCX-1777) has US orphan drug status for the treatment of non-Hodgkin's lymphoma, cutaneous T-cell lymphoma (CTCL), chronic lymphocytic leukemia (CLL) and related leukemias, including prolymphocytic leukemia (PLL), adult T-cell leukemia, hairy cell leukemia and acute lymphocytic leukemia (ALL). In December 2006, Forodesine was designed Orphan drug in Europe for ALL.

PNP inhibitors are also investigated to prevent the cleavage, and the resulting deactivation of Nucleoside Analogues by PNP.

**Description of the In vitro screening assay**

**PNP enzyme** used in the assay is a human recombinant PNP, cloned by NOVOCIB from human cells, expressed in *E. coli*, and produced and purified in NOVOCIB's laboratory. (see sheet # E-Nov 2 for further information) PNP purification is controlled before every assay by SDS-PAGE. Protein concentration is measured by Bradford method. PNP specific activity is then determined - 1 unit of PNP catalyzes the cleavage of 1 µmole of inosine per minute at pH 8.0 at 25 °C.

**Procedure**

NOVOCIB has developed a spectrophotometric procedure to directly follow the PNP phosphorolytic reaction on inosine (IR).

The assays are performed at 25°C or 37°C in 200µl of reaction buffer on 96-well microplate. Pipetting is done by a Multiprobe II Robotic Liquid Handling System (Packard BioScience). Ganciclovir is used as positive control for PNP inhibition. Replicate assays: One point is defined as a well per compound and per concentration. Ganciclovir is used as positive control for PNP inhibition. Controls are done in duplicate. Triplicates are available upon request.

Every assay, from one to 90 points, is done with one negative control, and two positive controls containing Ganciclovir as a PNP inhibitor. Controls are done in duplicate. If an additional microplate is needed, it includes the three controls (in duplicate, i.e. 6 wells).

**Confirmation by HPLC:**

For every positive assay, an HPLC (Agilent 1100 series) control of PNP inhibition is performed by measuring inosine (IR) and Hypoxanthine (Hx) concentrations in the assay and in comparison with negative and positive controls.

**Note:** Protozoan parasites are auxotrophic for purine and have their own PNP's which have specific activities and properties that differ from the human PNP. Protozoan parasites PNP's are considered to be reasonable target against infection (e.g. Plasmodium falciparum).

---

PNP Cleaving activity - Nucleoside Resistance Assay

**Aim:** To evaluate the resistance of Nucleoside Analogues to cleavage by human PNP.

**Nucleoside Analogues deactivation by PNP**

In *vivo*, phosphorolysis is highly favoured over purine nucleoside synthesis and is coupled with two additional enzymatic reactions: oxidation of the liberated purine base by xanthine oxidase (XO) and its phosphorosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Thus, PNP plays a key role in the salvage pathway of the purine metabolism, enabling the cell to utilize purine bases recovered from metabolized purine ribo- and deoxyribonucleosides to synthesize purine nucleotides.

This phosphorolysis reaction of purine nucleosides by PNP has a direct impact on the therapeutic efficacy of Nucleoside Analogues. Antitumour or antiviral nucleoside analogues are likely to be cleaved by PNP before being phosphorylated by the cell nucleoside kinases and converted to the active nucleotide form. For instance, 2',3'-dideoxyguanosine (ddG), 9-β-D-arabinofuranosyl guanine (AraG) as well as one of its produgs, Nelarabine (Arranon®, GSK) and 2',3'-dideoxynosine (ddl) is easily cleaved *in vivo* by PNP.

Since acyclonucleoside analogues are particularly resistant to cleavage by PNP though phosphorylated by viral thymidine kinases (TK), they are generally considered as excellent candidates as antiviral agents (e.g. aciclovir, ganciclovir).

Note that Ganciclovir is not only PNP resistant, but is also a PNP inhibitor.

NOVO CIB’s has developed a PNP enzymatic assay which consists in evaluating the PNP phosphorolysis activity on Nucleosides Analogue in comparison with natural purine nucleoside substrates.

**Description of the Nucleoside Resistance assay**

The assays are performed at 25°C or 37°C in 200µl of reaction buffer (lower volumes are available if compound saving is a constraint) on 96-well microplate. Pipetting is done by a Multiprobe® II Robotic Liquid Handling System (Packard BioScience).

Inosine is used as a positive control and adenosine as a negative control.

Note that if inosine is a natural substrate of PNP and is consequently actively cleaved by a wide range of PNP, adenosine resists to Human PNP phosphorolytic activity but is easily cleaved by other PNPs, such as E.coli PNP for instance.

This is why we consider as a decisive advantage to evaluate the cleavage resistance of Nucleoside Analogue using Human PNP.

Identification and quantification of the purine nucleosides and the related purine bases produced by Human PNP cleaving activity are performed by HPLC.

3. L. G. Gravatt et al. (1993): Efficacy and toxicity of 9-β-D-arabinofuranosylguanine (araG) as an agent to purge malignant T-cells from murine bone marrow: application to an in vivo T-cell leukemia model, Leukemia 7(8), 1261-1267
Nucleoside kinases, rate-limiting step of nucleoside analogues activation

Nucleoside analogues have proven to be a highly successful class of anti-cancer and anti-viral drugs. The therapeutic efficacy of nucleoside analogues is dependent on their intracellular phosphorylation. Two cellular nucleoside kinases, deoxycytidine kinase (dCK) and UMP-CMP kinase (YMPK) are critical for phosphorylation of cytidine analogues. These kinases provide two first steps of activation of highly effective anti-cancer and anti-viral drugs, such as 1-β-D-arabinofuranosylcytosine (araC, aracytidine), 2',2'difluorodeoxycytidine (dFdC, gemcitabine), β-D-2'3'-dideoxycytidine (ddC). Both kinases phosphorylate unnatural L-nucleosides (e.g., β-L-2'3'-dideoxy-3'thiacytidine, L-SSdC, 3-TC or lamivudine). Kinetic constants of araC, dFdC and 3TC phosphorylation by recombinant dCK and UMP-CMPK have been published. The comparison of phosphorylation properties of new nucleoside analogues to those of these known drugs provides the rational basis for selection of analogues of better therapeutic potential.

To characterize phosphorylation properties of new nucleoside analogues, NovoCIB has developed human recombinant dCK and human recombinant YMPK nucleoside phosphorylation assays. As shown in the Table 1, YMPK assay must be performed with monophosphate forms of nucleoside analogues and requires preliminary phosphorylation of nucleoside analogues and their purification. To circumvent this time-consuming step, Novocib has developed coupled dCK-YMPK nucleoside phosphorylation assay that delivers in one step the critical information on both dCK and YMPK substrate properties of nucleoside analogue.

Ribavirin (1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analogue with broad spectrum antiviral activity. Since 1970s it is known that the initial step of ribavirin phosphorylation is provided by adenosine kinase. Recently it has been demonstrated that cytosolic 5' nucleotidase II can also phosphorylate ribavirin, that could contribute to the development of ribavirin-induced haemolytic anemia in vivo. NovoCIB has developed both human recombinant adenosine kinase and cytosolic nucleotidase II nucleoside phosphorylation assays to evaluate properties of new ribonucleoside analogues in comparison with those of ribavirin.

Table 1. Available nucleoside kinase assays and reference nucleoside substrates

<table>
<thead>
<tr>
<th>Natural substrates</th>
<th>dCK assay</th>
<th>YMPK assay</th>
<th>Coupled dCK-YMPK assay</th>
<th>AK assay</th>
<th>5’cN-II assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyadenosine</td>
<td>dCMP, UMP, CMP</td>
<td></td>
<td></td>
<td>Adenosine</td>
<td></td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td></td>
<td></td>
<td></td>
<td>Inosine</td>
<td></td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td></td>
<td></td>
<td></td>
<td>Inosine</td>
<td></td>
</tr>
<tr>
<td>Cytidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleoside analogues substrates</td>
<td>Cladribine, fludarabine, Gemcitabine, Lamivudine, Aracytidine, Fluorodeoxyuridine</td>
<td>dFdCMP, araCMP, 3TCMP</td>
<td>Adefovir (9-(2-phosphonomethoxyethyl) adenine</td>
<td>Gemcitabine, Aracytidine, Lamivudine, Fluorodeoxyuridine</td>
<td>Ribavirin, Tubercidin, Mizoribin, Dideoxyinosine, Ribavirin, Acyclovir</td>
</tr>
</tbody>
</table>

1. R. C. Willis, D. A. Carson, and J. E. Seegmiller (1973) Adenosine Kinase Initiates the Major Route of Ribavirin Activation in a Cultured Human Cell Line. PNAS USA, 75: 3042-3044
Human deoxycytidine kinase (dCK)

Human, recombinant expressed in E.coli

E.C. 2.7.1.74

Description
NOVOCIB’s human deoxycytidine kinase (dCK) is a recombinant protein of ca.33kDa cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in E.coli.

Human deoxycytidine kinase plays a key role in the salvage pathway of deoxynucleotides synthesis providing resting cells with deoxynucleotides for DNA repair and mitochondrial DNA synthesis. The enzyme has a broad substrate specificity and provides the phosphorylation of both purine and pyrimidine deoxynucleosides (e.g. deoxyadenosine (dA), deoxyguanosine (dG)) and deoxycytidine (dC) and pyrimidine ribonucleoside, cytidine (C). The enzyme can utilize both ATP and UTP as phosphate donor with UTP being preferred substrate.

Deoxycytidine kinase is responsible for the phosphorylation and activation of numerous nucleoside analogs used to treat cancer (e.g. cytarabine, gemcitabine, cladribine and fludarabine) including nucleoside analogs of non-physiological L-chirality (e.g. 3TC, lamivudine, anti-HIV and anti-hepatitis B agent). Three-dimensional structures of dCK in complex with various pyrimidine and purine D- and L-nucleosides have been solved providing structural basis for activation of L- and D-nucleoside analogs.

Storage: −20 ° C in a solution containing 50 mM Tris-HCl, pH 7.6, 1 mM β-mercaptoethanol, 50% glycerol.

Unit Definition: One unit of deoxycytidine kinase converts 1.0 µmole of deoxycytidine and ATP to dCMP and ADP per minute at pH 7.6 at 37°C, as measured by a coupled PK/LDH enzyme system.

Specific Activity: ≥ 0.025 unit/mg protein.

Purity: controlled by 12%AA SDS-PAGE.

The enzymatic activity of human recombinant dCK was confirmed by ion-pair HPLC analysis (Agilent 1100 series, Zorbax C18plus) as shown by formation of dCMP and ADP (red) from deoxycytidine and ATP (blue).

Assay condition: Enzymatic activity of dCK is measured by spectrophotometric assays in a coupled lactate dehydrogenase/pyruvate kinase assay system. Assays were carried out at 37°C, at 50mM Tris-HCl pH7.6; 50mM KCl, 10mM MgCl2, 5mM ATP, 0.1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK 10U/ml, LDH 15U/ml, 0.9µM dCK. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm. The nucleosides, nucleotides, LDH and PK are purchased from Sigma-Aldrich.

Related products:

• dCK nucleoside phosphorylation assay
• Coupled dCK-YMPK nucleoside phosphorylation assays
• Coupled Nucleoside Kinase – IMPDH II
• UMP-CMP kinase (YMPK)
• Adenosine kinase (AK)
• Cytosolic 5’ nucleotidase II (nN-II)
• YMPK nucleotide monophosphate phosphorylation assay
• Adenosine kinase phosphorylation assay
• cN-II phosphorylation assay

**dCK nucleoside phosphorylation assay**

**Aim:** To characterize substrate properties (Km and Vmax) of new nucleoside analogues for human deoxycytidine kinase in comparison with those of known nucleoside analogues (e.g., aracytidine, gemcitabine, cladribine and lamivudine).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km,µM</th>
<th>Vmax, µmol/mg/min</th>
<th>Relative Vmax, % of dCR</th>
<th>Published data</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycytidine</td>
<td>0.577</td>
<td>0.026</td>
<td>100</td>
<td></td>
<td>Recombinant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Johansson Karlsson 1995</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.16, 0.033</td>
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<td>1.3, 0.069</td>
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<td></td>
<td></td>
<td></td>
<td>0.57, 0.004</td>
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</tr>
<tr>
<td>Gemcitabine</td>
<td>42.71</td>
<td>0.325</td>
<td>1250</td>
<td></td>
<td>Recombinant</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>150.5</td>
<td>1.08</td>
<td>4153</td>
<td></td>
<td>Sabini E et al 2008</td>
</tr>
<tr>
<td>Aracytidine</td>
<td>6.81</td>
<td>0.224</td>
<td>862</td>
<td></td>
<td>Recombinant</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Johansson Karlsson 1995</td>
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<td></td>
<td>115, 1.500</td>
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<td>480, 1.500</td>
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<td>89, 0.126</td>
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<td></td>
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<td>24, 0.76</td>
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</tr>
<tr>
<td>Cladribine</td>
<td>56.5</td>
<td>0.285</td>
<td>1096</td>
<td></td>
<td>Usova &amp; Eriksson, 1997</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Enzyme:** The dCK used in the assays is a human recombinant dCK, cloned from human cells, expressed in *E. coli*, produced and purified by **NOVOCIB** (see sheet # E-Nov 3 for further information). The enzyme purity is controlled before every assay by SDS-PAGE. Protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure dCK enzymatic activity (≥ 0.025 unit/mg protein).

**Kinetics Analysis:** Enzymatic activity of deoxycytidine kinase with particular nucleoside substrate is measured continuously by spectrophotometric assays in a coupled lactate dehydrogenase/pyruvate kinase system. Assays are carried out at 37°C, at 50mM Tris-HCl pH7.6; 50 mM KCl, 10mM MgCl2, 5mM ATP, 0.1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK 10U/ml, LDH 15U/ml, 0.9µM dCK. The nucleosides, nucleotides, LDH and PK are purchased from Sigma-Aldrich. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm. Assays are performed in duplicate (2 wells per compound and per concentration). Triplicates are available upon request. Km and Vmax are calculated from spectroscopic data using Michaelis-Menten equation.

A confirmation by HPLC analysis of formation of monophosphorylated forms is available upon request.

**Related products:**

- **Deoxycytidine kinase (dCK)**
- **Coupled dCK-YMPK nucleoside phosphorylation assays**
- **Coupled Nucleoside Kinase – IMPDH II**

**Related products:**

- **UMP-CMP kinase (YMPK)**
- **Adenosine kinase (AK)**
- **Cytosolic 5' nucleotidase II (cN-II)**
- **YMPK nucleotide monophosphate phosphorylation assay**
- **Adenosine kinase phosphorylation assay**
- **cN-II phosphorylation assay**

---

**NOVOCIB** has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE® services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.
**UMP-CMP kinase (YMPK)**

**Human, recombinant expressed in E.coli**

E.C. 2.7.4.14

**Synonyms:** YMPK, UMP/CMPK, UCK, CMK, CMPK, UMK, UMPK

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**Description**

NOVOCIB’s Human UMP-CMP kinase (YMPK) is a recombinant protein of ca. 27kDa (full length 228-aa form) cloned by RT-PCR amplification of mRNA extracted from Huh7 cells (human hepatoma) and expressed in *E.coli*.

UMP-CMP kinase plays a critical role in supplying cells with nucleotides by catalysing the phosphorylation of CMP, UMP and dCMP to their respective diphosphates. YMPK plays also an important role in the activation of cytidine analogues, aracytidine and gemcitabine, a mainstay of leukaemia and lymphoma therapy. YMPK has a remarkable ability of to phosphorylate L-nucleotides from their monophosphate to diphosphate forms as shown for β-L-2’3’-dideoxy-3’thiacytidine (L-SSdC, 3-TC or lamivudine), an anti-HIV and anti-hepatitis B drug.

Crystal structure of open form of human UMP-CMP kinase has been solved recently. These data together with homology model of enzyme in closed state provides structural basis for understanding the substrate specificity of the enzyme and helps to design of new nucleoside analogues of higher phosphorylation efficiency.

**Storage:** –20 °C in a solution containing 150 mM KCl, 50 mM Tris-Hcl, pH 7.5, 2 mM β-mercaptotethanol, 50% glycerol.

**Unit Definition:** One unit of UMP-CMP kinase converts 1.0 µmole of UMP and ATP to UDP and ADP per minute at pH 7.6 at 25°C, using a coupled enzyme system with PK/LDH.

**Specific activity:** ≥ 0.150U/mg

**Purity:** controlled by SDS-PAGE

---

**Related products:**

NOVOCIB has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE® services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.

- **UMP-CMP kinase (YMPK) nucleoside phosphorylation assay**
- **Coupled dCK-YMPK nucleoside phosphorylation assays**
- **Deoxycytidine kinase (dCK)**
- **Adenosine kinase (AK)**
- **Cytosolic 5’ nucleotidase II (cN-II)**
- **dCK nucleoside phosphorylation assay**
- **Adenosine kinase phosphorylation assay**
- **cN-II phosphorylation assay**
- **Coupled Nucleoside Kinase – IMPDH II**

---

YMPK nucleotide monophosphate phosphorylation assay

Aim: To characterize substrate properties (Km and Vmax) of monophosphate forms of new nucleoside analogues for human YMPK in comparison with monophosphate forms of natural nucleosides or reference nucleoside analogues.

Enzyme: The enzyme used in the assays is a human recombinant YMPK, cloned from human cells, expressed in E. coli, produced and purified by NOVOCIB (see sheet # E-Nov 4 for further information). The enzyme purity is controlled before every assay by SDS-PAGE. Protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure YMPK enzymatic activity (≥ 0.150 unit/mg protein).

The enzyme used in the assays is a human recombinant YMPK, cloned from human cells, expressed in E. coli, produced and purified by NOVOCIB (see sheet # E-Nov 4 for further information). The enzyme purity is controlled before every assay by SDS-PAGE. Protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure YMPK enzymatic activity (≥ 0.150 unit/mg protein).

<table>
<thead>
<tr>
<th>Km, µM</th>
<th>Vmax, nmol/mg/min</th>
<th>Relative Vmax, % of CMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP 17,9</td>
<td>130,07</td>
<td>100</td>
</tr>
<tr>
<td>UMP 392</td>
<td>307,16</td>
<td>236</td>
</tr>
<tr>
<td>dCMP 1334</td>
<td>297,65</td>
<td>228</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Km, µM</th>
<th>Vmax, nmol/mg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP 17,95</td>
<td>130,07</td>
</tr>
<tr>
<td>UMP 392</td>
<td>307,16</td>
</tr>
<tr>
<td>dCMP 1334</td>
<td>297,65</td>
</tr>
</tbody>
</table>

Kinetics Analysis: Substrate properties of particular nucleoside monophosphate for YMPK are evaluated in a continuous LDH/PK spectrophotometric assay. The assays are carried out at 37°C, at 50mM Tris-HCl pH 7,6; 50mM KCl, 10mM MgCl2, 5mM ATP, 0,1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK 10U/ml, LDH 15U/ml, YMPK. The nucleosides, nucleotides, LDH and PK are purchased from Sigma-Aldrich. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm. Assays are performed in duplicate (2 wells per compound and per concentration). Triplicates are available upon request. Km and Vmax are calculated from spectrophotometric data using Michaelis-Menten equation.

A confirmation by HPLC analysis of formation of monophosphorylated forms is available upon request.

Related products: NOVOCIB® has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE® services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.

- UMP-CMP kinase (YMPK)
- Coupled dCK-YMPK nucleoside phosphorylation assays
- Deoxycytidine kinase (dCK)
- Adenosine kinase (AK)
- Cytosolic 5’ nucleotidase II (cN-II)
- dCK nucleoside phosphorylation assay
- Adenosine kinase phosphorylation assay
- cN-II phosphorylation assay
- Coupled Nucleoside Kinase – IMPDH II

References
Coupled dCK-YMPK nucleoside phosphorylation assays

**Aim:** Coupled dCK-YMPK nucleoside phosphorylation assay is a cost-effective rapid assay that delivers in one step the critical information on both dCK and YMPK substrate properties of nucleoside analogue.

**Enzymes:** The enzymes used in this assay are human recombinant dCK and human recombinant YMPK, cloned by NovoCIB from human cells, expressed in *E. coli*, and produced and purified by NOVOCIB (see sheet # E-Nov 3 and # E-Nov 4 for detailed information).

**Method validation:**

The phosphorylation kinetic of CMP by recombinant YMPK have been measured in two independent approaches. In first one, YMPK Km for CMP was studied directly with CMP substrate (grey), and in second one YMPK Km for CMP was measured indirectly in coupled dCK-YMPK assay (red) using cytidine as a substrate. As shown on left, coupled dCK-YMPK assay produce the results highly similar to those of direct YMPK assay.

**Related products:**

- UMP-CMP kinase (YMPK) nucleoside phosphorylation assay
- dCK nucleoside phosphorylation assay
- UMP-CMP kinase (YMPK)
- Deoxyxycytidine kinase (dCK)
- Adenosine kinase (AK)
- Cytosolic 5’ nucleotidase II (cN-II)
- Adenosine kinase phosphorylation assay
- cN-II phosphorylation assay
- Coupled Nucleoside Kinase – IMPDH II
Human adenosine kinase (AK)

Human, recombinant expressed in E.coli

EC 2.7.1.20

Synonyms: AK, ADK

Description

NOVOCIB’s human adenosine kinase (AK) is a recombinant protein of ca.39kDa (345-aa short form8) cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in E.coli. The sequence of the cloned AK (GenBank accession number US50196) was confirmed by DNA sequencing (100% identity).

Adenosine kinase is a ubiquitous enzyme that catalyzes the transfer of γ-phosphate from ATP to 5’ hydroxyl of adenosine generating AMP and ADP. Adenosine (AR) is an important modulator of central nervous system functions with a half-life of seconds. Facilitated diffusion of adenosine across the cell membrane closely couples adenosine concentrations in the intracellular and extracellular compartments. Inhibition of adenosine kinase results in selective increase of local adenosine concentrations and reduced seizure susceptibility and nociception in vivo9. Adenosine kinase is an attractive and experimentally validated target for the development of new analgesic and anti-inflammatory agents4. In addition, recently AK has emerged as a novel target to predict and to prevent epileptogenesis5, 6. The X-ray crystallographic structure of human AK has been described7 and provides structural basis for rational design and optimisation of new AK inhibitors.

In addition, this enzyme is responsible for the phosphorylation and consequent clinical activity of several therapeutically useful nucleosides, including the antiviral drug ribavirin8, immunosuppressive drug mizoribine9 and anticancer C-nucleoside, tiazofurin10.

Storage: −20 °C in a solution containing 50 mM Tris-HCl, pH 7.6, 1 mM β-mercaptoethanol, 50% glycerol.

Unit Definition: One unit of adenosine kinase converts 1.0 μmole of adenosine and ATP to AMP and ADP per minute at pH 7.6 at 30°C, as measured by a coupled PK/LDH enzyme system.

Specific Activity: ≥ 0.030 unit/mg protein.

Purity: controlled by 10% AA SDS-PAGE.

Adenosine kinase phosphorylation assay

Adenosine Km = 11.4μM
Deoxyadenosine Km = 269μM
Inosine Km = 1757μM

Assay condition: Enzymatic activity of adenosine kinase with particular nucleoside substrate is measured by spectrophotometric assays in a coupled lactate dehydrogenase / pyruvate kinase system. Assays were carried out at 37°C, at 50mM Tris-HCl pH 7.6, 50mM KCl, 5mM MgCl2, 2.5mM ATP, 0.1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK 50μl, LDH 50μl. Reaction is followed in an iEMS Reader MF (LabSystems) microtiter plate reader at 340nm. The nucleosides, nucleotides, LDH and PK are purchased from Sigma-Aldrich.

Related products:

NOVOCIB has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE® services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.

3. ABT-702 (4-Amino-5-(3-bromophenyl)-7-(6-morpholino pyridin-3-yl)pyrido[2,3-
4. Cytosolic 5’ nucleotidase II (cN-II)
5. YMPK nucleotide monophosphate phosphorylation assay
cN-II phosphorylation assay
AK nucleoside phosphorylation assay

IMPORTANT: Client-specified alterations can be accommodated.

**Aim:** To characterize substrate properties (Km and Vmax) of new nucleoside analogues for human adenosine kinase in comparison with those of known nucleoside analogues (e.g., ribavirine, tubercidine or mizoribine).

<table>
<thead>
<tr>
<th>Substrat</th>
<th>Km (µM)</th>
<th>Kcat (min⁻¹)</th>
<th>Km (µM)</th>
<th>Kcat (min⁻¹)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>11</td>
<td>1.5</td>
<td>3.2</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.150</td>
<td></td>
<td>2</td>
</tr>
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<td>Ribavirine</td>
<td>328</td>
<td>1.9</td>
<td>540</td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>295</td>
<td>3.4</td>
<td>360</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Tubercidine</td>
<td>12</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>1758</td>
<td>2.6</td>
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</table>

**Enzyme:** The AK used in the assays is a human recombinant AK, cloned from human cells, expressed in E. coli, produced and purified by NOVOCIB (see sheet # E-Nov5 for further information). The enzyme purity is controlled by SDS-PAGE. Protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure AK enzymatic activity (≥ 0.030 unit/mg protein).

**Kinetics Analysis:** Enzymatic activity of adenosine kinase with particular nucleoside substrate is measured continuously by spectrophotometric assays in a coupled lactate dehydrogenase/pyruvate kinase system. Assays are carried out at 37°C, at 50mM Tris-HCl pH7.6; 50mM KCl, 5mM MgCl₂, 2.5mM ATP, 0.1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK-LDH (5U/ml each), 0.85µM AK. The nucleosides, nucleotides, LDH and PK are purchased from Sigma-Aldrich. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm. Assays are performed in duplicate (2 wells per compound and per concentration). Triplicates are available upon request. Km and Vmax are calculated from spectroscopic data using Michaelis-Menten equation. A confirmation by HPLC analysis of formation of monophosphorylated forms is available upon request.

**Related products:**

NOVOCIB has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE® services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.

- Adenosine kinase
- Coupled Nucleoside Kinase – IMPDH II
- Deoxycytidine kinase (dCK)
- UMP-CMP kinase (YMPK)
- Cytosolic 5’ nucleotidase II (cN-II)
- YMPK nucleotide monophosphate phosphorylation assay
- dCK nucleoside phosphorylation assay
- Coupled dCK-YMPK nucleoside phosphorylation assays
- cN-II phosphorylation assay

**References:**

Human cytosolic 5’-nucleotidase II (cN-II)

*Human, recombinant expressed in E.coli*

**EC 3.1.3.5**

**Synonyms:** High Km 5’-NT, purine 5’-NT, IMP-GMP-specific 5’-NT

**Description**

NOVOCIB’s human cytosolic IMP/GMP specific 5’-nucleotidase/phosphotransferase II (cN-II) is a recombinant protein of ca.65kDa cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in E.coli. The sequence of the cloned CN-II (GenBank accession number P49902) was confirmed by DNA sequencing (100% identity).

Cytosolic 5’-nucleotidase II is one of the seven known mammalian nucleotidases\(^1\) that specifically catalyzes the dephosphorylation of 6-hydroxypurine nucleoside 5’-monophosphates (IMP, dIMP, GMP, dGMP) and regulates cellular pool of IMP and GMP\(^2,3\). The enzyme also acts as a phosphotransferase catalyzing the transfer of a phosphate from nucleoside monophosphate to a nucleoside acceptor – preferentially inosine and deoxyinosine. Unlike the other 5’-nucleotidases, cN-II is allosterically regulated by adenine/guanine nucleotides and 2,3-biphosphoglycerate\(^4\).

In addition, cytosolic 5’-nucleotidase II phosphorylates anti-viral and anti-tumour nucleoside analogues such as 2’3’-dideoxyinosine, carbovir\(^5\), acyclovir\(^6\) and ribavirin\(^7\).

**Storage:** –20 °C in a solution containing 50 mM Tris-HCl, pH 7.6, 2 mM β-mercaptoethanol, 50% glycerol.

**Unit Definition:**

One unit of 5’nucleotidase converts 1.0 µmole of IMP to inosine per minute at pH 7.6 at 37°C, as measured by a coupled PNP/XO enzyme system in the presence of 20 mM MgCl\(_2\), 5mMDTT, 500µM KH\(_2\)PO\(_4\), and 1.25mM IMP.

**Specific Activity:**

≥ 0.150 unit/mg protein.

**Purity:** controlled by 10% AA SDS-PAGE.

**5’nucleotidase assay condition:** 5’nucleotidase activity of cN-II is followed in an irreversible spectrophotometric assay using coupled purine nucleoside phosphorylase - xanthine oxidase system (2.5mU/ml each). Assays were carried out at 37°C, at 50mM Tris-HCl pH7.6; 100mM KCl, 20mM MgCl\(_2\), 500µM KH\(_2\)PO\(_4\), 5mM DTT, 119nM cN-II and various concentration of IMP. Reaction is followed at 295nm. The IMP is is purchased from MPBiomedicals, XO is from Sigma-Aldrich and PNP is from Novocib (ref ENov-2).

**Related products:**

NOVOCIB has cloned and purified a panel of human recombinant nucleoside kinases and has developed a range of PRECICE\(^\text{®}\) services to evaluate substrate properties of new nucleoside analogues for key cellular kinases.

- cN-II phosphorylation assay
- Coupled Nucleoside Kinase – IMPDH II
- Adenosine kinase
- Deoxycytidine kinase (dCK)
- UMP-CMP kinase (YMPK)
- dCK nucleoside phosphorylation assay
- YMPK nucleotide monophosphate phosphorylation assay
- Coupled dCK-YMPK nucleoside phosphorylation assays
- Adenosine kinase nucleoside phosphorylation assays

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\(^3\) Ipata PL, Tozzi MG Recent advances in structure and function of cytosolic IMP-GMP specific 5’-nucleotidase II (2006) Purinergic Signal. 2(4):669-75


IMP Dehydrogenase (IMPDH, E.C. 1.1.1.205) catalyzes the pivotal step in guanine nucleotide biosynthesis, the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), and controls the guanine nucleotide pool. A number of nucleoside analogues (e.g. ribavirin, mizoribine) are known to inhibit IMPDH after being monophosphorylated. The therapeutic consequences of IMPDH inhibition vary for different analogues - mizoribine is an immunosuppressor and ribavirin is a broad spectrum antiviral. Even if direct relationship between ribavirin antiviral action and IMPDH inhibition by ribavirin monophosphate has not been demonstrated, the depletion of cellular GTP might result in an increased frequency of ribavirin triphosphate incorporation by viral polymerase due to a lower intracellular concentration of its natural competitor.

Aim: For rapid evaluation of monophosphate forms of nucleoside analogues as IMPDH inhibitors.

<table>
<thead>
<tr>
<th>dCK</th>
<th>AK</th>
<th>5’cN-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural substrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>Adenosine</td>
<td>Deoxyinosine</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>Inosine</td>
<td>Inosine</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleoside analogues substrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>Ribavirin</td>
<td>Dideoxyinosine</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>Tubercidin</td>
<td>Ribavirin</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>Mizzoribine</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>Lamivudine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aracytidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluordeoxyuridine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzymes: The monophosphorylation step of nucleoside analogue is provided by one of the specific human recombinant nucleoside kinases: AK (ref. # E-Nov 5), dCK (ref. # E-Nov 3), cN-II (ref. # E-Nov 6) produced by NOVOCIB. Human recombinant IMPDH 2 was cloned from human cells, expressed in E. coli and purified by NOVOCIB (see sheet # E-Nov 1 for further information). The enzyme purity is controlled by SDS-PAGE, protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure enzymatic activity.

**References**


2) L.J. Stuyver, S. Lostia, S.E. Patterson, J.L. Clark, K. A. Watanabe, M.J. Otto and K.W. Parkewicz (2002) Inhibitors of the IMPDH enzyme as potential antivirals viral diarrhoea virus agents Antiviral Chemistry & Chemotherapy 13:345–352

**Phosphorylation of nucleoside analogue is provided by specific nucleoside kinase and confirmed by spectrophotometric quantification of formed ADP in LDH-PK coupled reaction.**

**IMPDH inhibition:** Effect of monophosphorylated nucleoside analogues on human recombinant IMPDH II. Enzymatic assays performed in duplicate are carried out at 37°C in 0.1M KH2PO4 buffer pH 8.0 in the presence of 2mMDTT, 200µM NAD, 200µM IMP and 0.2 µM IMPDH II and increasing concentration of monophosphorylated nucleoside. Reaction is followed in an iEMS Reader MF (Labsystems) microtiter plate reader at 340nm.

**Related products:**

- dCK nucleoside phosphorylation assay
- Adenosine kinase nucleoside phosphorylation assays
- cN-II phosphorylation assay
- Deoxycytidine kinase (dCK)
- Adenosine kinase
- Cytosolic 5’ nucleotidase II (cN-II)
- UMP-CMP kinase (YMPK)
- YMPK nucleotide monophosphate phosphorylation assay
- Coupled dCK-YMPK nucleoside phosphorylation assays
IMPDH - Whole Cell Assay

IMPORTANT: Client-specified alterations can be accommodated.

Aim
This service has been specially tailored to validate IMPDH inhibition by a given compound in cultured cells. This whole cell assay consists in extracting, identifying and quantifying by HPLC the intracellular concentration of guanosine nucleotides (GMP, GDP and GTP) and IMP in compound-treated cells. This service was validated with mycophenolic acid, ribavirin and mizoribin, recognized inhibitors of IMPDH. When applied for the study of nucleoside analogues (NA), this assay can also reveal the formation of their mono-, di-, and triphosphate forms, indicating that nucleoside analogues enter the cells and are readily phosphorylated by cellular kinases.

1st Example: Mycophenolic acid (MPA)
As illustrated by Figure 1, a 48h-incubation of Huh 7 cells with mycophenolic acid (Sigma-Aldrich, 5µM), a known inhibitor of cellular IMPDH, results in a dramatic depletion of cellular GTP. As expected, the intracellular concentration of GMP is lowered, while IMP concentration is increased. Table 1 and Figure 2 present results of quantification of nucleotide mono- and tri-phosphates in treated and untreated cells.

Figure 1. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.

Figure 2. Effects of 5µM MPA on cellular pool of nucleotide mono- and di-phosphates (results of quantification of HPLC spectra presented on Figure 1)

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>DMSO 0.125%</th>
<th>MPA 5µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>280.2</td>
<td>233.2</td>
</tr>
<tr>
<td>UMP</td>
<td>102.2</td>
<td>100.2</td>
</tr>
<tr>
<td>GMP</td>
<td>33.8</td>
<td>9.6</td>
</tr>
<tr>
<td>IMP</td>
<td>27.0</td>
<td>137.2</td>
</tr>
<tr>
<td>AMP</td>
<td>396.6</td>
<td>273.2</td>
</tr>
<tr>
<td>CTP</td>
<td>554.0</td>
<td>424.8</td>
</tr>
<tr>
<td>GTP</td>
<td>1,237.0</td>
<td>182.0</td>
</tr>
<tr>
<td>UTP</td>
<td>1,734.8</td>
<td>1,627.0</td>
</tr>
<tr>
<td>ATP</td>
<td>7,665.0</td>
<td>7,057.0</td>
</tr>
</tbody>
</table>
**IC₅₀ determination:** Cellular GTP concentrations are plotted as a function of inhibitor concentration. IC₅₀ is calculated using a standard four-parameter nonlinear regression analysis. Plotting of minor nucleotides, such as IMP and GMP, is also available upon request.

**2nd Example: Ribavirine (Rbv)**
Numerous nucleoside analogues (NA) are currently used to treat viral infections. They are usually designed to inhibit one viral target. This remains in contrast with the observation that ribavirin, a purine nucleoside analogue currently used as a part of bi-therapy of hepatitis C infection, has multiple modes of action: (i) depletion of intracellular GTP pools by inhibition of the cellular IMPDH, (ii) inhibition of viral polymerase activity, (iii) induction of error catastrophe as a result of accumulation of mutations in the viral genome. Even if direct relationship between ribavirin antiviral action and IMPDH inhibition has not been demonstrated, the depletion of cellular GTP should result in increased frequency of ribavirin triphosphate incorporation by viral polymerase due to lower intracellular concentration of its natural competitor.

To study the effect of nucleoside analogues on whole spectra of cellular purine and pyrimidine ribo- and deoxyribonucleotides, we have developed original cell-based analytical approach in which more than 31 (deoxy)ribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pared chromatography and quantified. This cellular assay was validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, lefunomide, hydroxyurea). In regards with new antiviral molecules identified in HCV cell culture systems (e.g. replicon), our cell-based assay allows to select the molecules of direct antiviral action from inhibitors of cell nucleotide biosynthesis.

**Figure 3.** Modifications in cell-pool of nucleotides in Ribavirin-treated cells

**Figure 4.** Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 10µM Rbv (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.
Materials & Methods

Cells treatment
Huh-7 cells are grown in an atmosphere of humidified 5% CO\textsubscript{2} at 37° C in DMEM medium supplemented with 2mM L-glutamine, 10% heat-inactivated fetal bovine serum and streptomycin-penicillin. Exponentially grown Huh-7 cells are seeded at ~6x10\textsuperscript{5} cells per 10cm cell-culture dish. After 48h of growth, the culture medium is replaced with fresh FCS-supplemented medium followed by addition of 10µL of DMSO or DMSO-dissolved compound.

Extraction of nucleotides and deoxynucleotides - Sample preparation
The nucleotides are extracted from cell monolayers by addition of 3 ml per dish of ice-cold 80% acetonitrile for 1h. The extracts are centrifuged to remove cellular debris and nucleotides are extracted by SPE procedure (SAX column, Supelco, Sigma-Aldrich) preconditioned with methanol, water and acetonitrile. The eluent is filtered through 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system
1) An Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostatted column compartment G1316A and multiple wavelength and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent). 5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides\textsuperscript{1} with slight modifications as follows.

HPLC calibration, peak identification and quantification
Calibrations are performed with standards prepared in mobile phase and with standards mixed with cell extracts, which are run immediately before and after every series of samples. Assignment of the peaks that correspond to different deoxyribonucleoside and ribonucleoside mono-, di-, and triphosphate of the cell extract spectrum is done by comparing both retention times and characteristics of UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

Aim
This service has been specially tailored to validate RNR inhibition by a given compound in cultured cells. This whole cell assay consists in extracting, identifying and quantifying by HPLC the intracellular concentration of deoxynucleotides di- and triphosphate in compound-treated cells. This service was validated with hydroxyurea and gemcitabine in HeLa cultured cells.

1st Example: Hydroxyurea (HU)

**Figure 1**: Nucleotide profiles of hydroxyurea-treated HeLa cells

**Hydroxyurea** is an antineoplastic agent, anti metabolite, used to treat melanoma, chronic myelocytic leukemia and certain blood disorders. Hydroxyurea is known to inhibit DNA synthesis by destroying the catalytically essential free radical of class I ribonucleoside diphosphate (rNDP) reductase, thereby blocking the de novo synthesis of deoxyribonucleotides. In mammalian cells, hydroxyurea treatment causes a differential depletion of the four deoxyribonucleoside triphosphate pools with dATP being most severely depleted. As illustrated by Figure 1, hydroxyurea treatment induces in HeLa cells profound depletion of deoxyadenosine triphosphate and significant loss of dADP, dUDP and dTTP, which is consistent with previously published data.

**Figure 2**: Effect of hydroxyurea on cellular pool of deoxynucleotides. The depleted nucleotides are shown in red. Ribonucleotide reductase (RNR), a recognized target of hydroxyurea, is framed in red.
Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and dTTP, dATP on right.

2nd Example: Gemcitabine (dFdC)

Nucleotide profiles of Gmc-treated HeLa cells (37µM, 20h)

Ratio between nucleotide content in drug-treated and untreated cells are shown.

Figure 4: Nucleotide profiles of gemcitabine-treated HeLa cells

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Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a nucleoside analogue clinically used as an anticancer prodrug. Its phosphorylated metabolites target numerous cellular enzymes involved in nucleotide biosynthesis, including ribonucleotide reductase (RNR) which is strongly inhibited by diphosphorylated form of gemcitabine, dFdCDP. As shown in Figure 4, major changes in nucleotides induced by dFdC in HeLa cells concern the depletion in cellular dATP, dTTP, dGTP and dUDP due to RNR inhibition. The depletion of cellular dUMP indicates inhibition of dCMP-deaminase consistently with previously reported data\(^1\), but may also reflect the decrease in cellular dUDP, source of dUMP.

Figure 5. Effect of gemcitabine on cellular pool of nucleotides and deoxynucleotides. The depleted (<50% of control) nucleotides are shown in red. Ribonucleotide reductase (RNR) and dCMP-deaminase (dCMP-DA), recognized targets of gemcitabine are framed in red.

Figure 6. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 37µM gemcitabine (blue) and DMSO (red) illustrating depletion in dNTP.

Materials & Methods

Cells treatment:
HeLa cells were grown in an atmosphere of humidified 5% CO₂ at 37°C in DMEM (PAA) medium supplemented with 2mM L-glutamine (Gibco/BRL), non essential amino acids (PAA), 10% heat-inactivated fetal bovine serum (BioWest) and streptomycin-penicillin (Sigma). Exponentially grown HeLa cells were seeded at ~6x10⁵ cells per dish. After 48h of growth, the culture medium was replaced with fresh FCS-supplemented medium (10ml per Petri dish) followed by addition of 10µL of DMSC or DMSO-dissolved compounds. Six Petri dishes of cells per experiment were used to provide the nucleotide amount sufficient for UV-quantification of deoxynucleotides. At the end of a 7h-incubation, the medium was aspirated, cells monolayers washed twice with 5ml PBS, and used for nucleotides extraction.

Extraction of nucleotides and deoxynucleotides - Sample preparation: The nucleotides were extracted from cell monolayers by the addition of ice-cold 80% acetonitril for 1h. The extracts were centrifuged to remove cellular debris and load on SAX column (100mg, Supelco) pre-conditioned with methanol, water and acetonitrile. Once sample was effused completely, the cartridge was washed with 3ml 80% ACN and 3ml water and eluted with 1M KCl. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system:
1) Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and multiple wavelength and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).
5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides¹ with slight modifications as follows. Peak identification of the different nucleoside mono-, di-, and triphosphates, was made from their characteristic UV absorption spectra and retention times compared with those of a mixture of standards (Sigma) run immediately before cell extracts. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

HPLC conditions:
Nucleotides were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications in pH and concentration of buffers adjusted to ensure adequate resolution of all nucleosides/nucleotides as follows: Buffer A: 20mM KH₂PO₄, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH₂PO₄, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature constantly kept at 21°C. Gradient was formed as follows: 15 min at 10.0% buffer A; 5min at up to 90% buffer A, 5 min up to 70% buffer A; 15min up to 63% buffer A, 20min up to 45% buffer A, 10min up to 25% buffer A, 10min up to 0% buffer A. The spectra were recorded at 254 and 280nm.

HPLC calibration:
The calibration was performed with following standards: dUMP, dUDP, dUTP, dCDP, dCMP, dCDP, dCTP, dTMP, dTTP, dGDP and dGTP were not separated from unknown major peak and were not quantified. The standards prepared in Buffer A or those mixed with cell extracts were run immediately before and after series of samples. The data were used for calculation of retention times (Rf) and absorbance at 254nm and 280nm (254/280 ratio) specific for each nucleotide.

Peak identification and quantification:
5µl of cell extract were injected and nucleotides were separated as described before. Assignment of peak of the different deoxyribonucleosides and ribonucleosides mono-, di-, and triphosphate was done by comparing both retention times and characteristic UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

Quality control:
The experiments are done in duplicates and relative standard deviation (RSD) is usually less than 12%.

Multi-targeted Antifolates - Whole Cell Assay

Inhibition of de novo purine and pyrimidine biosynthesis

Aim
This service has been specially tailored to validate inhibition of de novo biosynthesis of purine and pyrimidine nucleotides by a given compound in cultured cells. After incubation of cultured cells with the inhibitor, nucleotides are extracted, separated, identified and quantified by UV-HPLC. This service was validated with methotrexate and HeLa cultured cells.

Methotrexate (MTX) is an immunosuppressive agent that has been in clinical use for over 50 years. Although originally introduced for chemotherapy in cancer and leukaemia, MTX was coincidentally found to have immunosuppressive properties and is currently used in treating rheumatoid arthritis. MTX was first believed to be an inhibitor of the enzyme dihydrofolate reductase (DHFR), the enzyme required for reduction of dihydrofolate (FH$_2$) to tetrahydrofolate (FH$_4$). However, as shown in the 80's, MTX is actually a prodrug which is polyglutamated and accumulated in cells. In contrast to unmodified MTX, its polyglutamated derivatives were found to be efficient inhibitors of the ninth folate-dependent step of purine synthesis catalysed by 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate transformylase (AICAR-T) and the thymidylate synthetase (TS).

**Figure 1:** Nucleotide profiles of methotrexate-treated HeLa cells

**Figure 2:** Effect of MTX on cellular pool of purines and pyrimidines.
Results:
As illustrated by Figures 1 and 3, intracellular level of ATP, ADP, GTP and GDP is much lower in methotrexate-treated cells than in untreated control, while cellular contents of UTP and UDP are not affected. Another remarkable change concerns the accumulation of dUMP in methotrexate-treated HeLa cell and depletion of dTTP pool. All these results are in perfect agreement with previously published data showing that MTX inhibits \textit{de novo} synthesis of purine nucleotides through AICART enzyme and synthesis of thymidylate through thymidylate synthase.

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**Materials & Methods**

**Cells treatment:**
HeLa cells were grown in an atmosphere of humidified 5% CO\textsubscript{2} at 37°C in DMEM (PAA) medium supplemented with 2mM L-glutamine (Gibco/BRL), non essential amino acids (PAA), 10% heat-inactivated fetal bovine serum (BioWest) and streptomycin-penicillin (Sigma). Exponentially grown HeLa cells were seeded at ~6x10\textsuperscript{5} cells per dish. After 48h of growth, the culture medium was replaced with fresh FCS-supplemented medium (10ml per Petri dish) followed by addition of 10µL of DMSC or DMSC-dissolved compounds. Six Petri dishes of cells per experiment were used to provide the nucleotide amount sufficient for UV-quantification of deoxynucleotides. At the end of a 7h-incubation, the medium was aspirated, cells monolayers washed twice with 5ml PBS, and used for nucleotides extraction.

**Extraction of nucleotides and deoxynucleotides - Sample preparation:**
The nucleotides were extracted from cell monolayers by the addition of ice-cold 80% acetonitril for 1h. The extracts were centrifuged to remove cellular debris and load on SAX column (100mg, Supelco) pre-conditioned with methanol, water and acetonitrile. Once sample was effused completely, the cartridge was washed with 3ml 80% ACN and 3ml water and eluted with 1M KCl. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

**Analytical system:**
1) Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.

2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).

5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications as follows:

Peak identification of the different nucleoside mono-, di-, and triphosphates, was made from their characteristic UV absorption spectra and retention times compared with those of a mixture of standards (Sigma) run immediately before cell extracts. The area of individual peaks was measured using ChemStation software (Agilent).

**HPLC conditions:**
Nucleotides were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications in pH and concentration of buffers adjusted to ensure adequate resolution of all nucleotides/nucleotides as follows: Buffer A: 20mM KH\textsubscript{2}PO\textsubscript{4}, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH\textsubscript{2}PO\textsubscript{4}, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature constantly kept at 21°C. Gradient was formed as follows: 15 min at 100% buffer A; 5min at up to 90% buffer A, 5 min up to 70% buffer A; 15min up to 63% buffer A, 15 min up to 55% buffer A, 20min up to 45% buffer A, 10min up to 25% buffer A, 10min up to 0% buffer A. The spectra were recorded at 254 and 280nm.

**HPLC calibration:**
The calibration was performed with following standards: dUMP, dUDP, dUTP, dCDP, dCMP, dCDP, dCTP, dTMP, dTTP, dGDP and dGTP were not separated from unknown major peak and were not quantified. The standards prepared in Buffer A or those mixed with cell extracts were run immediately before and after series of samples. The data were used for calculation of retention times (Rf) and absorbance at 254nm and 280nm (254/280 ratio) specific for each nucleotide.

Peak identification and quantification:
5µl of cell extract were injected and nucleotides were separated as described before. Assignment of peak of the different deoxyribonucleosides and ribonucleosides mono-, di-, and triphosphate was done by comparing both retention times and characteristic UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

**Quality control:**
The experiments are done in duplicates and relative standard deviation (RSD) is usually less than 12%.

---

Aims: To analyze the Nucleoside & Nucleotide content in biological samples

From a one-compound analysis (e.g. ADP, ATP, NAD, NADP...) to a whole spectrum of nucleosides and nucleotides.

A wide range of samples can be analyzed: cultured cells, blood cells, body fluids...

Do not hesitate to contact us for any matter of feasibility!

This service is intended for many purposes, including the analysis of nucleotides in cultured cells for which it has been specially developed to study the drug impact on the cell metabolism of nucleosides and nucleotides in comparison with untreated cells.

In this case:
- it enables to:
  • reveal the metabolic changes due to the drug action, either on the purine or on the pyrimidine pathway,
  • identify the metabolites whose levels are modified by the drug treatment,
  • trace back to the metabolic step(s) altered by the drug, and to its likely target(s).
- cell culture and treatment procedures are the followings: The choice of the cell line and culture conditions has been optimized to get highly reproducible results. Assays are usually done with human hepatoma cell line Huh7. Cells are grown in DMEM supplemented with FCS (5%), glutamine (1mM), sodium pyruvate (1mM) and maintained in exponential phase. Cells are seeded on 10cm-dishes and allowed to adhere overnight. The drug is added next day at the agreed concentration and at a cell confluence of about 50%.

The following metabolites are routinely analyzed:

<table>
<thead>
<tr>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>NAD</th>
<th>NADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>GDP</td>
<td>GMP</td>
<td>IMP</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>UDP</td>
<td>UDP-glucose</td>
<td>UMP</td>
<td>CTP</td>
</tr>
<tr>
<td>NADH</td>
<td>NADPH</td>
<td>dATP</td>
<td>dGTP</td>
<td>dTTP</td>
</tr>
<tr>
<td>Guanine</td>
<td>Uracyl</td>
<td>Hypoxanthine</td>
<td>Inosine</td>
<td>Uridine</td>
</tr>
<tr>
<td>Cytosine</td>
<td>Cytidine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Some of them, depending on the sample, are naturally present at trace level (e.g. IMP, hypoxanthine). However, they can be detected and quantified in appropriate larger samples or when their accumulation is due to the drug action (e.g. under treatment by mycophenolic acid, an IMPDH inhibitor, as shown below).

Sample size:

Cultured cell: Nucleosides & Nucleotides Analysis is usually performed by extraction of ~10^7 treated cells, per compound and per concentration tested. Control untreated cells are cultured under the same conditions to provide a reference metabolic profile. Depending on the cell line or the experimental conditions, a 0.5-1.10^5 cell-extract can be sufficient to analyze the major metabolites (e.g. ATP, ADP...).

Blood cells: typically, a 200µl-sample of blood is sufficient to analyze the major metabolites in Red Blood Cells (RBC), and a 1ml-sample for Peripheral Blood Mononuclear cells (PBMC). If needed, for instance to focus on naturally low-level metabolites, larger samples can be prepared, e.g. up to 10^9 of cultured cells.

Nucleosides & nucleotides separation and analysis: The extraction and separation procedures have been optimized and specially developed by NOVOCIB. After a 48h-treatment, nucleosides and nucleotides are extracted; Nucleosides, nucleotides mono-, di-, and triphosphates, deoxynucleotides triphosphates and bases are separated by ion-pairing HPLC (Agilent 1100) with a Zorbax EclipsePlus C18 column and quantified using an Agilent ChemStation software. The resulting values are normalized by cell number. A mixture of 30 authentic standards (Sigma Aldrich, Roth) is run before and after every set of samples analysis.

Separation by ion-pairing HPLC of a 6.6pmol / 20µl standard mixture of 30 nucleosides, nucleotide mono-, di-, and triphosphates, deoxynucleotide triphosphates and bases
Quantification of intracellular metabolites in Huh-7 non-treated cells

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>pmol / 10^6 cells</th>
<th>published data</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMP</td>
<td>728.1 +/- 56.8</td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>34.4 +/- 4.8</td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>72.7 +/- 21.4</td>
<td>(130)</td>
</tr>
<tr>
<td>NAD</td>
<td>1,825.1 +/- 155.5</td>
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</tr>
<tr>
<td>UDPglu</td>
<td>1,113.9 +/- 87.1</td>
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</tr>
<tr>
<td>AMP</td>
<td>122.7 +/- 12.9</td>
<td></td>
</tr>
<tr>
<td>UDP</td>
<td>1,730.3 +/- 207.1</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>2,754.5 +/- 165.4</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>4,652.4 +/- 608.8</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>2,494.2 +/- 156.1</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>1,449.7 +/- 81.4</td>
<td>(1540)</td>
</tr>
<tr>
<td>ATP</td>
<td>6,561.0 +/- 346.0</td>
<td>(6580)</td>
</tr>
</tbody>
</table>

* Mean +/- SD for 5 independent experiments

** J. Balzarini et al. (1993): Eicar (5-ethyl-1-β-D-ribofuranosylimidazole-4-carboxamide): a novel potent inhibitor of inosinate dehydrogenase activity and guanylate biosynthesis J. Biol. Chem. 268 (33), 24591–24598

Metabolic signatures of mycophenolic acid, an IMPDH (inosine monophosphate dehydrogenase) inhibitor (left) and of leflunomide, a DHODH (dehydroorotate dehydrogenase) inhibitor (right) obtained in Huh-7 cultured cells.

For every metabolite, drug-treated / non-treated cells concentration ratios are calculated and graphically reported. The base line indicates the control level.

Metabolism and activation of nucleoside analogues: The separation and analytical procedures developed by NOVOCIB are particularly relevant to study the cellular metabolism of nucleoside analogues. The phosphorylation step by cellular kinases is crucial for the activation of nucleoside analogues and their efficacy.

At NOVOCIB, we routinely measure ribavirine and ribavirine mono-, di-, and tri-phosphate in cell culture extract, serum and blood cells. Quantification of other mono-, di- and triphosphorylated nucleoside analogues can be performed (subject to feasibility).

Please, see the Cellular Pharmacology of Nucleoside Analogues information sheet.

IP Rights: Only drugs available on the market are used to build up our metabolic profiles database. Every result obtained from the metabolic profile of any compound supplied by our client will remain its full ownership. Every related piece of information will be considered confidential and will not be transmitted to any other party.
**Aims:** To identify and quantify, in cell extract, a nucleoside analogue drug and its derivatives produced by the cell metabolism.

Cell permeability, target specificity and drug metabolism are crucial cellular parameters upon which the drug efficiency depends. The cell metabolism of a drug is of major importance in the case of nucleoside analogues since most, if not all, of them act as prodrugs. To be active, once entered the cell, nucleoside analogues have to be phosphorylated by cell kinases. Mono-, di- and tri-phosphate nucleotide forms, as well as other possible cell-modified derivatives (e.g. deamination) of the corresponding nucleoside analogue act differently on the cell metabolism. (see references).

Our cellular pharmacology service is usually performed on cultured cell extract but its analytical component can be applied to other biological samples for pharmacokinetics studies.

**Nucleotides and nucleosides Analysis:** The separation and analytical procedures developed by NOVO CIB are particularly relevant to study the cellular pharmacology of nucleoside analogues. They have been optimized for several nucleoside analogues, particularly with ribavirin. However, the separation and analytical procedures must be specifically adapted to every nucleoside analogue.

**Cell culture and treatment:** The choice of the cell line and culture conditions has been optimized to get highly reproducible results. Assays are usually done with human hepatoma cell line Huh7. Cells are grown in DMEM supplemented with FCS (5%), glutamine (1mM), sodium pyruvate (1mM) and maintained in exponential phase. Cells are seeded on 10cm-dishes and allowed to adhere overnight. The drug is added next day at the agreed concentration and at a cell confluence of about 50%.

Other samples than cultured cells can be analyzed: blood cells, body fluid...

Do not hesitate to contact us for any matter of feasibility!

**Separation of gemcitabine (dFdC) and gemcitabine di- (dFdC-DP) and triphosphate (dFdC-TP) in Huh7 cell extract:**
- Blue: non-treated cells
- Red: 48h-treatment with gemcitabine (dFdC) at 200 µM
- * dFdC-MP was not detected in this experiment

**Separation of ribavirin (Rbv) and ribavirin mono- (Rbv-MP), di- (Rbv-DP) and triphosphate (Rbv-TP) in Huh7 cell extract:**
- Blue: non-treated cells
- Red: 48h-treatment with ribavirin at 3 mM

**Separation of cytarabine (AraC) and cytarabine mono- (AraC-MP), di- (AraC-DP) and triphosphate (AraC-TP) in Huh7 cell extract:**
- Blue: non-treated cells
- Green: 48h-treatment with cytarabine (AraC) at 260 µM
- Red: 48h-treatment with cytarabine (AraC) at 130 µM

**References**


E. Mini, S. Nobili, B. Caciagli, I. Landini & T. Mazzoli (May 2006): Cellular pharmacology of gemcitabine J. Oncol. 17(6), v7-v12


**K value measurement for the determination of Fish & Seafood freshness**

**Enzymatic method or HPLC method**

**Aim:** To determine the level of freshness of foodstuffs, especially for fish and seafood.

**The K value concept**

In the late 1950’s, a Japanese research team (Saito et al.) proposed a new concept, called "K value", for the indication of the freshness of fish. It is based on ATP breakdown due to respiratory cell death, and the subsequent formation of nucleotide by-products, namely inosine monophosphate (IMP), inosine (Ino) and, at a later stage, hypoxanthine (Hx).

K-value measures how far ATP degradation has progressed within a tissue. It is expressed as a percentage of the content of the last two final compounds of the ATP catabolic pathway, i.e. "Ino" and "Hx", over the total content of ATP and its degradation products, that is to say ATP, ADP, AMP, IMP, Ino and Hx.

However, because ATP decomposes very quickly to IMP in most animals, a simplified K value (generally called K value) was soon proposed by Karube et al. (1984) and is currently considered as equivalent to the original 6-parameter equation for K-value:

\[
K = \frac{[\text{Ino}] + [\text{Hx}]}{[\text{IMP}] + [\text{Ino}] + [\text{Hx}]} 
\]

The lower the K value, the fresher the flesh.

In the value chain of fish and seafood industry, K-value is particularly relevant at the early stages of storage and processing, during autolysis and before spoilage starts. From a time-window point of view, K-value measurement is highly complementary, without redundancy, to other Quality Control analytic tools such as TVB-N, TMA... which are pertinent at a later stage of the foodstuff lifespan. In other words, K-value allows any professional to anticipate the beginning of spoilage that other usual controls measure.

**NOVOCIB** has developed an enzymatic system to measure the K value, which is considered for several decades as the most effective and objective indicator of the freshness of fish, as well as of meat (beef, pork, lamb and chicken).

**Fish sample preparation**

About 1g of muscle (preferably dorsal muscle for finfish) is collected and heated in boiling water for 2-4 minutes.

**Extraction**

Once homogenized, nucleotides are extracted according a protocol we have optimized on the basis of the one routinely applied for the analysis of nucleotides in cultured cell (see sheet # Nucleotide Analysis).

**Measure**

Analysis is performed through enzymatic assays that have been validated by comparison with HPLC spectra (see picture: Determination coefficient was \( R^2=0.99 \)). HPLC method for K-value determinations are also available.

Please note that our enzymatic assay for K-value determination will soon be available as a kit. Do not hesitate to contact us if you want to be informed about its expected launching.

**Applications:**

- Freshness of Finfish, Shellfish (scallop), Crustaceans (shrimp, prawn, crab…), Cephalopods (squid, octopus…)
- Raw, chilled, frozen, smoked, salted, pickled, cooked seafood products
- K-value can be used on other meat-based foodstuffs: beef, lamb, pork, chicken…

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F. Özgül et al. (2008): Nucleotide degradation and biogenic amine formation of wild white grouper (Epinephelus aeneus) stored in ice and at chill temperature (4 °C), *Food Chem.* 106(3), 933–941


V. Lisada et al. (2005): Inhibition of chemical changes related to freshness loss during storage of horse mackerel (Trachurus trachurus) in slurry ice, *Food Chem.* 93(4), 619–625

N. Hamada-Sato et al. (2005): Quality assurance of raw fish based on HACCP concept, *Food Control* 16(4), 361–367


V.P. Lougovois et al. (2003): Comparison of selected methods of assessing freshness quality and remaining storage life of iced gilthead sea bream (Sparus aurata), *Food Res. Intern.* 36(6), 551–560


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**K value changes in Rainbow Trout**

[Graph showing K value changes in Rainbow Trout]
Aim: To determine the content of nucleotides monophosphate in baby milk powder, particularly CMP, UMP, GMP, IMP and AMP.

Nucleotides are naturally present in milk (i.e. 2-5% of the non-protein nitrogen in Human milk). However, after studies had suggested that nucleotides have positive effects in infant nutrition, manufacturers have got used to adding nucleotides in baby food, for several years now and particularly in milk powder. Unsurprisingly, in a context where food additives are more and more subjected to regulation, nucleotide additions in baby milk powders do or are about to enter under the control of strict rules.

Nucleotides additives in baby food are generally monophosphate nucleotides. NOVOCIB proposes to analyze CMP, UMP, GMP, IMP and AMP contents in baby milk. Other nucleotides analysis are available.

Nucleosides & nucleotides separation and analysis: The extraction procedure of nucleotides has been optimized for milk powder analysis. It involves a specific extraction protocol from the powder matrix, a fixation-washing-elution step on a strong anion exchange (SAX, Supelco) cartridge and a filtration through a 0.45µm filter membrane (Roth).

Separation method is based on what is routinely performed for cell extract analysis (ref sheet # NA for further information), with slight adjustments of buffers pH and concentrations. Nucleotides are separated by ion-pairing HPLC (Agilent 1100) system with a Zorbax EclipsePlus C18 column and quantified using an Agilent ChemStation software.

Linearity - Limit of Detection
Nucleotides monophosphate (NMP) standards are from Sigma-Aldrich, MPBiochemicals or Roche. 1mg/ml NMP stock solution is prepared in water and kept in the freezer (-20°C). Working standards are prepared daily at concentrations of 0.25, 0.5, 1.25, 2.5, 5, 10, 25 and 37.5 mg/100 g by dilution of stock solution with mobile phase A. The limit of detection is defined as the concentration where RSD (relative standard deviation) is <20%. A typical chromatogram of 5 nucleotide standards is shown in Figure 1. The related linearity and LOD results are shown in table 1.

Table 1. Linearity and LOD of NMP

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$R^2$</th>
<th>LOD (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>0.9989</td>
<td>0.025</td>
</tr>
<tr>
<td>AMP</td>
<td>0.9990</td>
<td>0.025</td>
</tr>
<tr>
<td>IMP</td>
<td>0.9922</td>
<td>0.050</td>
</tr>
<tr>
<td>UMP</td>
<td>0.9877</td>
<td>0.025</td>
</tr>
<tr>
<td>GMP</td>
<td>0.9990</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Recovery and Reproducibility
The extraction of milk powder samples are done with and without spiked NMP (2.5mg of each NMP per 100g powder) for every provided sample.

Table 2. Recovery of 5 spiked NMP’s in a baby milk powder (4 measurements)

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>77%</td>
<td>14%</td>
</tr>
<tr>
<td>UMP</td>
<td>94%</td>
<td>16%</td>
</tr>
<tr>
<td>GMP</td>
<td>89%</td>
<td>19%</td>
</tr>
<tr>
<td>IMP</td>
<td>85%</td>
<td>9%</td>
</tr>
<tr>
<td>AMP</td>
<td>97%</td>
<td>19%</td>
</tr>
</tbody>
</table>

Fig. 1. Superposed spectra of 5 concentrations of standards (0.25, 0.5, 1.25, 2.5 and 5mg/100g).

Fig. 2. Superposed chromatograms of milk powder without exogenous nucleotide (blue) and of a milk powder spiked with NMP at 2.5mg/100g (red).
