

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.



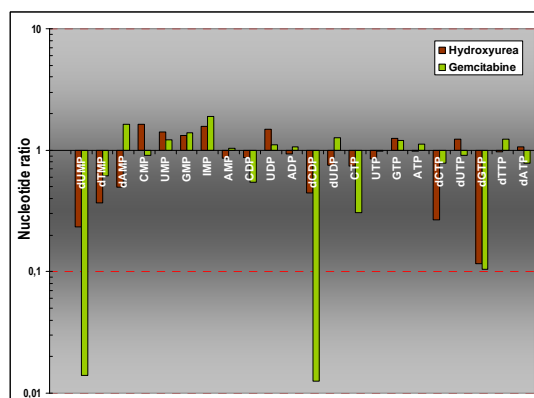
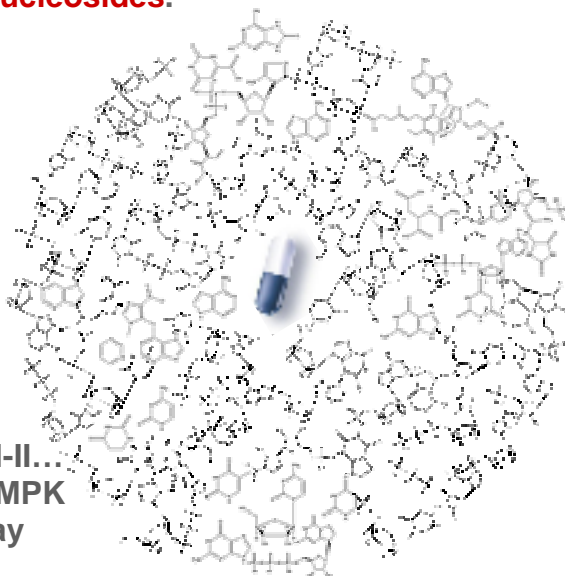
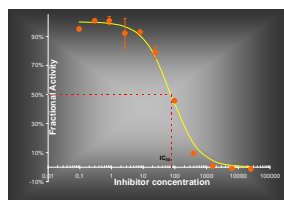
Nucleosides Metabolism Explorer

NOVO CIB 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, **NOVO CIB** 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
- Specificities 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 NOVOCIB.

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 *

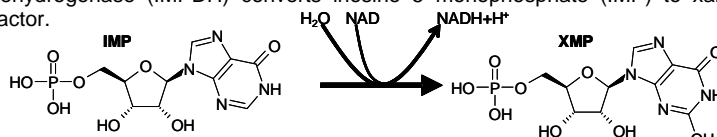
CNRS, Lyon, France
Université Claude Bernard, Lyon, France
Université de Clermont-Ferrand, France

* 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 processes¹. 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 differentiation². IMPDH is recognized as a validated target for several major therapeutic areas. IMPDH inhibitors are exploited as antiviral (e.g. ribavirin), antiparasitic, antimicrobial, antileukemic and immunosuppressive agents². IMPDH Type II is the predominant isoform of the enzyme and is selectively expressed in proliferating cells, including lymphocytes and tumor cells².

IMPDH in immunology

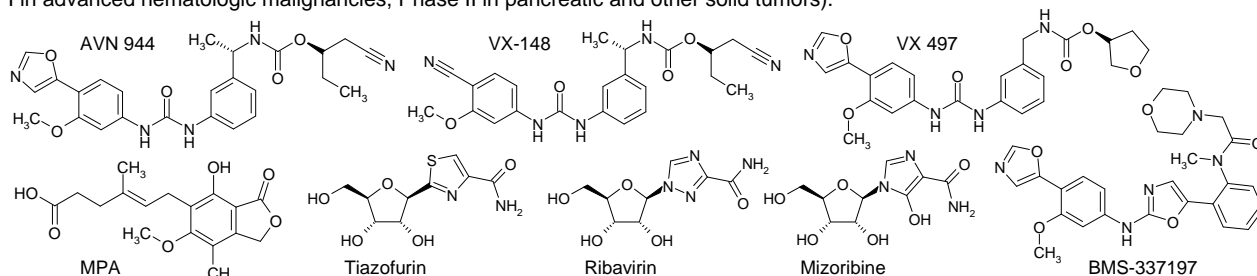
IMPDH is highly active in lymphocytes. It is a validated target to treat immunological diseases and to induce immunosuppression (CellCept®, a mycophenolic acid (MPA) prodrug - Roche - CHF1.85 Bn as an immunosuppressive agent in 2006, orphan drug designation in 2006 for Myasthenia Gravis, Phase III in Lupus Nephritis). IMPDH is also recognized as an excellent target for the treatment of psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)³.

IMPDH in oncology

IMPDH, and particularly Type II, which is overexpressed in tumor cells, is considered as a highly potent target for cancer chemotherapy^{1,2,4,5}. Several IMPDH inhibitors are under development for the treatment of Acute and Chronic Myelogenous Leukemia (AML, CML)⁶, and other cancers (pancreas, colon, bladder...). Additionally, it has been shown that the use of IMPDH inhibitors counteracts the drug resistance⁷ that may appear in certain tumors. For instance, methotrexate resistance is directly related to the overexpression of IMPDH, whose inhibition restores the drug efficacy⁸. 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 encouraging⁸. The IMPDH II atomic structure has been resolved and it provides a valuable background for further leads optimization⁹. Besides nucleosides analogues, NCEs have been identified as IMPDH inhibitors^{10,11,12,13,14} 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.

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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

NOVO CIB'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.

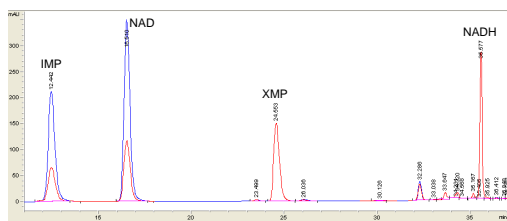
Unit Definition: One unit of IMPDH Type II catalyzes the formation of 1 µmole of NADH per minute at pH 8.0 at 25 °C

Specific Activity: \geq 0.035 unit/mg protein.

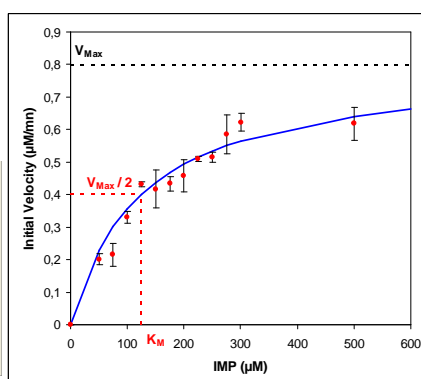
Assay condition: KH₂PO₄ 0.1M, pH7.8, NAD 180µM, DTT 1mM, 0.13mU of human recombinant IMPDH II (2µl at 0.081 U/mg protein)

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.

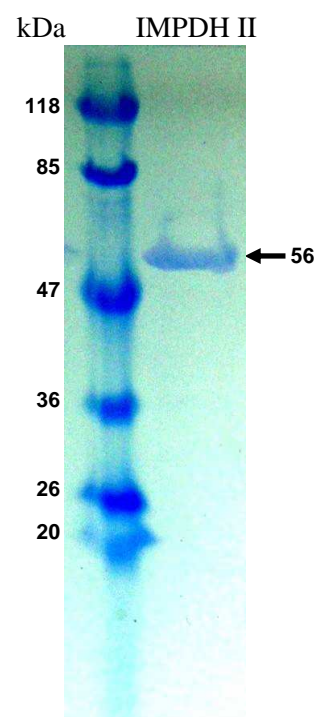
Purity controlled by SDS-PAGE



IMPDH activity was confirmed by HPLC analysis for quantification of IMP, XMP, NAD and NADH



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



IMPDH inhibition assays

NOVO CIB has cloned and purified a human recombinant Inosine Monophosphate Dehydrogenase, Type II (IMPDH II) and has developed a range of PRECICE® services to better evaluate the potential of compounds to inhibit IMPDH.

This key enzyme of nucleoside metabolism is recognized as a validated target to treat immunologic disorders, cancers and infectious diseases.

Applications: Chemical library screening,
Hit selection, Lead optimization
Complementary studies for drug development

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

IMPDH II - *In vitro* Assay

IMPORTANT: Client-specified alterations can be accommodated.

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 IC₅₀ 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 **NOVO**CIB'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 200 μl of reaction buffer on 96-well microplate. Reaction buffer is: KH₂PO₄ 0.1M, pH7.8, NAD 180 μM, DTT 1mM

Automation: Pipetting is done by a Multiprobe® 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 50 μM

- For Kinetics Analysis (IC₅₀): 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:

MPA (nM)	0,17	0,51	1,52	4,56	13,69	41,07	123,2	370	1109	3326	9980
log ₁₀	-0,77	-0,29	0,18	0,66	1,14	1,61	2,09	2,57	3,04	3,52	4,00

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.

Negative control	DMSO, without inhibitor
Positive controls	DMSO with MPA
Assay (11 points)	Compound to be tested at the concentrations indicated by the client
Incubation for 10mn with ~0.15 mU/well of human recombinant IMPDH II	
Reaction starts by adding IMP at 100 μM (final concentration)	

*Solubility in the reaction buffer must be checked before performing the assay.

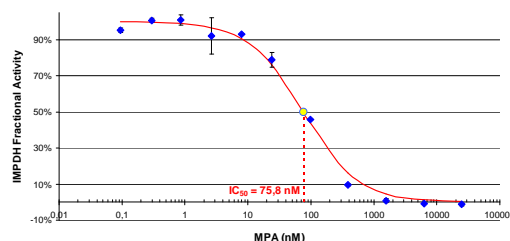
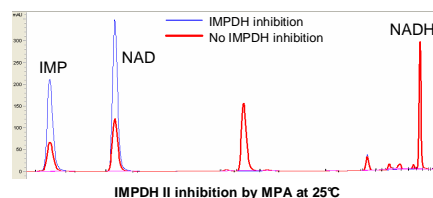
NADH formation is measured every mn for 30 mn in an iEMS Reader MF (Labsystems, Finland) microtiter plate reader at 340nm. Activity is determined by: $\Delta A / \epsilon_{\text{NADH}} \cdot p \cdot t$ where ϵ_{NADH} is the molar extinction coefficient for NADH at 340nm (= 6220 M⁻¹.cm⁻¹), ΔA is the absorbance variation at 340nm from t = 0 to t, p is the light pass in a well (= 0.625 cm for 200 μl/well), t is the maximal time (≤30mn) 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⁺ and NADH concentrations in the assay and by comparison with negative and positive controls.

For Kinetics Analysis, IC₅₀ 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. IC₅₀ 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 +/- 1 above the IC₅₀ 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...).

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

Description

NOVO CIB'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*.

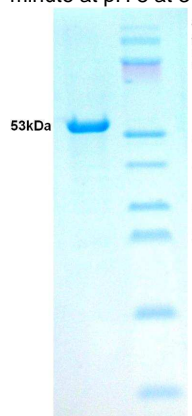
Today, antibiotic resistance is one of the world's most important public health problems. There is an urgent need for new antibiotic compounds acting on new targets. One attractive strategy for developing new antibiotics consists in inhibiting bacterial IMPDH, an enzyme involved in the *de novo* synthesis 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 **NOVO CIB** providing the tools for selection of species-specific IMPDH inhibitors.

Unit Definition:

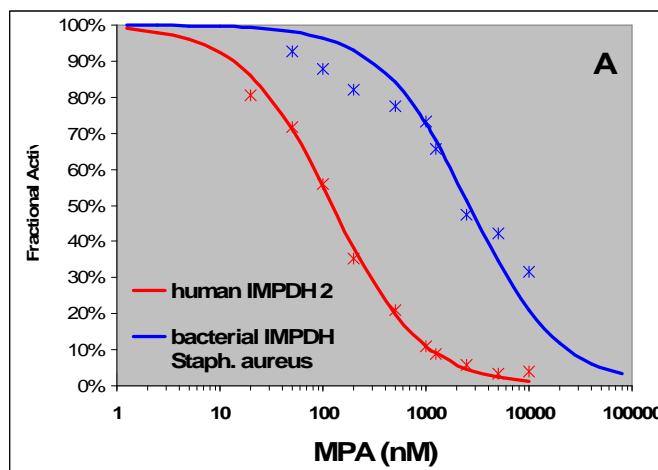
One unit of IMPDH converts 1.0 μmole of IMP and NAD to XMP and NADH per minute at pH 8 at 37°C.



Specific Activity:

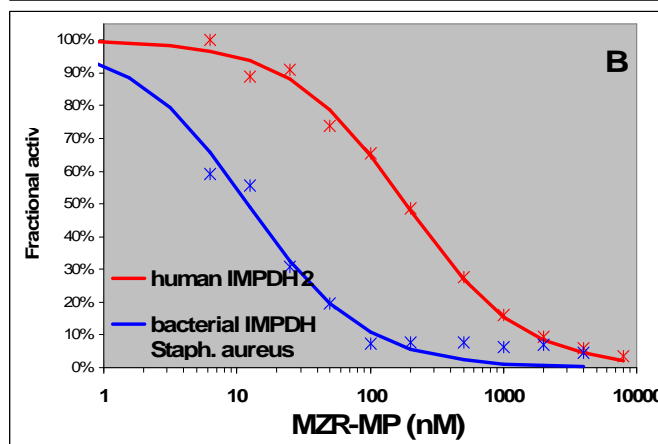
≥ 0.3 unit/mg protein.

Purity: controlled by 12%AA SDS-PAGE.



IMPDH 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).

References:

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Related products:

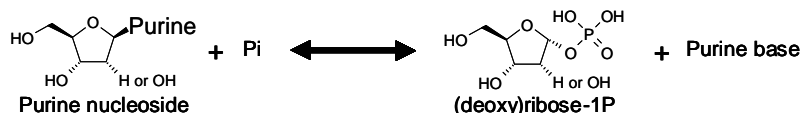
- **Coupled nucleoside kinase – IMPDH assay**
- **Human recombinant IMPDH II**
- **IMPDH II inhibition in vitro assay**
- **Human Adenosine kinase (AK)**

Purine Nucleoside Phosphorylase, a multiple-faced enzyme

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:

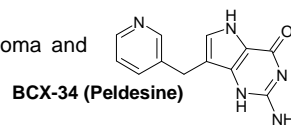


Therapeutic potential of PNP inhibitors

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^{1,2}. 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.

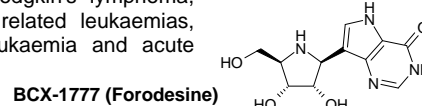
Some PNP inhibitors have undergone clinical trials for the treatment of cutaneous T-cell lymphoma, acute lymphoblastic leukemia (ALL), HIV infections, and psoriasis.

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 leukaemia (CLL) and related leukaemias, including prolymphocytic leukaemia (PLL), adult T-cell leukaemia, hairy cell leukaemia and acute lymphocytic leukaemia (ALL)³.

In December 2006, Forodesine was designed Orphan drug in Europe for ALL⁴.



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)⁵.

PNP, a threat for therapeutic efficacy of Nucleoside Analogues

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 phosphoribosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT)⁶. 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)⁷, 9-β-D-arabinofuranosyl guanine (AraG)⁸ as well as one of its produg, Nelarabine (Arranon®, GSK)⁹, which is intracellularly converted to AraG by Adenosine deaminase (ADA), are PNP resistant nucleoside analogues, whereas 2',3'-dideoxyinosine (ddI)¹⁰ 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)¹¹.

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")

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7. V. Gandhi *et al.* (1995): Cytotoxicity, metabolism, and mechanisms of action of 2',2'-difluorodeoxyguanosine in Chinese hamster ovary cells, *Cancer Res.* 55(7), 1517-1524
8. L. C. 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
9. C. U. Lambe *et al.* (1995): 6-Amino-6-methoxypurine arabinoside: an agent for T-cell malignancies, *Cancer Res.* 55(15), 3352-3356
10. M. Weibel (1994): Potentiating effect of [2[2-(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]ethenyl phosphonic acid (MDL 74,428), a potent inhibitor of purine nucleoside phosphorylase, on the antiretroviral activities of 2',3'-dideoxyinosine combined to ribavirin in mice, *Biochem. Pharmacol.* 48, 245-252
11. D. Shugar (1999): Viral and host-cell protein kinases: enticing antiviral targets, and relevance of nucleoside, and viral thymidine, kinases, *Pharmacol. Ther.* 82(2-3), 315-335

PNP - Purine Nucleoside Phosphorylase

Human, recombinant expressed in E. coli

E.C. 2.4.2.1

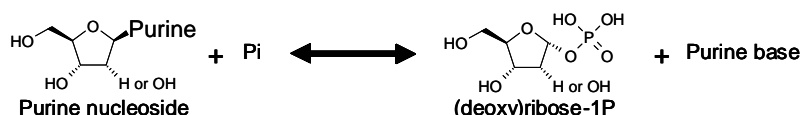
Description

Metabolic function

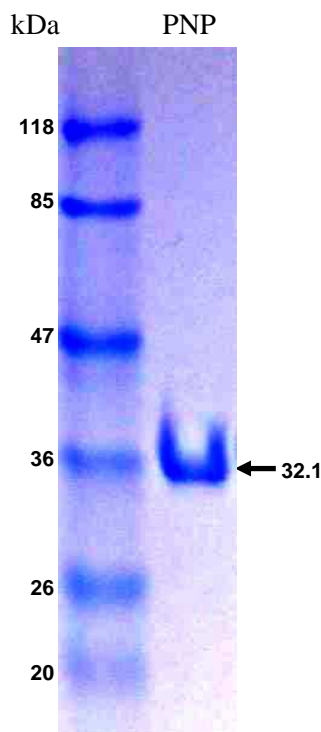
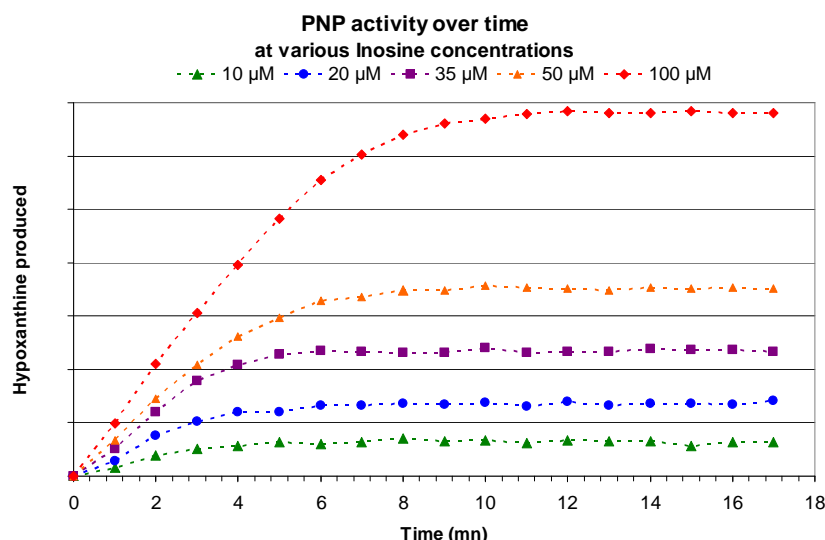
Purine Nucleoside Phosphorylase (PNP) is involved in salvage pathway of the purine metabolism.

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:



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.
(see sheet # IVS-Nov2, "PNP Inhibition - In vitro Screening Assay" for further details)

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.
(see sheet # NCR-Nov2, "PNP Cleavage activity - Nucleoside Resistance Assay" for further details)

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" for further details)

NOVO CIB has cloned and purified a human recombinant Purine Nucleoside Phosphorylase (PNP) and has developed a range of related PRECICE® services.

PNP Services

- PNP Inhibition - *In Vitro* Screening Assay
- PNP Cleavage activity - Nucleoside Resistance Assay
- (coming soon) Transribosylation by PNP

PNP Inhibition - *In vitro* Screening Assay

IMPORTANT: Client-specified alterations can be accommodated.

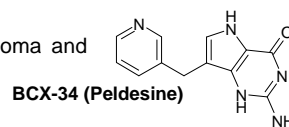
Aim: To screen compounds for their abilities to inhibit human PNP *in vitro*.

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^{1,2}. 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.

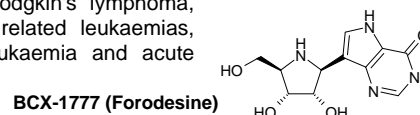
Some PNP inhibitors have undergone clinical trials for the treatment of cutaneous T-cell lymphoma, acute lymphoblastic leukemia (ALL), HIV infections, and psoriasis.

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 leukaemia (CLL) and related leukaemias, including prolymphocytic leukaemia (PLL), adult T-cell leukaemia, hairy cell leukaemia and acute lymphocytic leukaemia (ALL)³.

In December 2006, Forodesine was designed Orphan drug in Europe for ALL⁴.



PNP inhibitors are also investigated to prevent the cleavage, and the resulting deactivation of Nucleoside Analogues by PNP.
(see sheet # NCR-Nov2, "PNP Cleavage activity - Nucleoside Resistance Assay" for further details)

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*)⁵.

Description of the *In vitro* screening assay

PNP enzyme used in the assay is a human recombinant PNP, cloned by **NOVO CIB** from human cells, expressed in *E. coli*, and produced and purified in **NOVO CIB**'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 μ mole of inosine per minute at pH 8.0 at 25 °C

Procedure

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

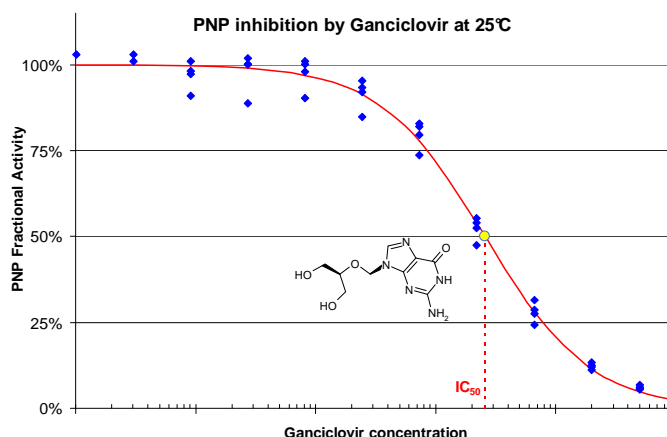
The assays are performed at 25°C or 37°C in 200 μ l of reaction buffer on 96-well microplate. Pipetting is done by a Multiprobe[®] II Robotic Liquid Handling System (Packard BioScience). Ganciclovir is used as positive control for PNP inhibition.

Replicate assays: One point is defined as a well per compound and per concentration tested. *In vitro* PNP Inhibition Screening Assays are usually performed in duplicate (2 wells per compound and per concentration). Both negative and positive controls are done in duplicate. Triplicates are available upon request.

Every assay, from one to 90 points, is done with one negative control, i.e. without inhibitor, 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.



1. S. Hikishima *et al.* (2007): **Synthesis and biological evaluation of 9-deazaguanine derivatives connected by a linker to difluoromethylene phosphonic acid as multi-substrate analogue inhibitors of PNP**, *Bioorg. Med. Chem. Lett.* 17(15) 4173-4177
2. T. Yokomatsu *et al.* (1999): **Synthesis and biological evaluation of 1,1-difluoro-2-(tetrahydro-3-furanyl)ethylphosphonic acids possessing a N9-purinylmethyl functional group at the ring, a new class of inhibitors for purine nucleoside phosphorylases**, *Bioorg. Med. Chem. Lett.* 9(19) 2833-2836
3. http://salesandmarketingnetwork.com/news_release.php?ID=2000113
4. <http://emea.europa.eu/pdfs/human/comp/opinion/45260406en.pdf>
5. K. Chaudhary *et al.* (2006): **Toxoplasma gondii Purine Nucleoside Phosphorylase Biochemical Characterization, Inhibitor Profiles, and Comparison with the Plasmodium falciparum Ortholog**, *J. Biol. Chem.* 281(35), 25652-25658

PNP Cleaving activity - Nucleoside Resistance Assay

IMPORTANT: Client-specified alterations can be accommodated.

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 phosphoribosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT)¹. 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)², 9-β-D-arabinofuranosyl guanine (AraG)³ as well as one of its produg, Nelarabine (Arranon®, GSK)⁴, which is intracellularly converted to AraG by Adenosine deaminase (ADA), are PNP resistant nucleoside analogues, whereas 2',3'-dideoxyinosine (ddI)⁵ 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)⁶.

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 Analogues in comparison with natural purine nucleoside substrates.

Description of the Nucleoside Resistance assay

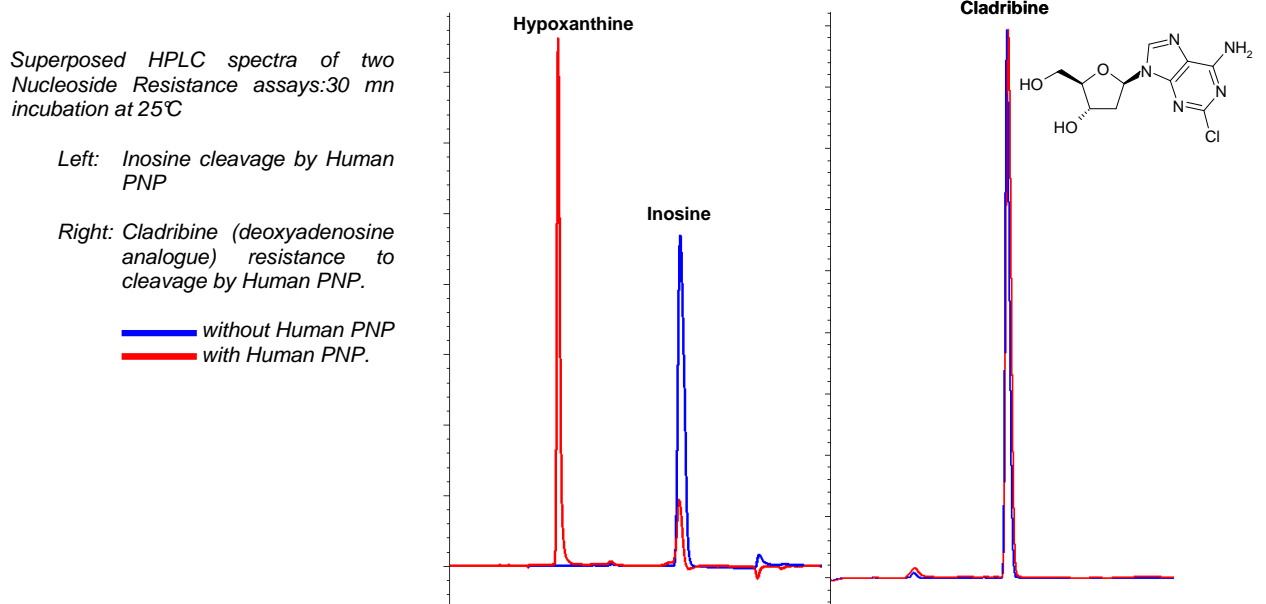
The assays are performed at 25°C or 37°C in 200µl of reaction buffer (lower volumes are available if compound saving is a constraint) on 96-well microplate. Pipetting is done by a Multiprobe® II Robotic Liquid Handling System (Packard BioScience).

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 PNPs, 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.



1. A. Bzowska *et al.* (2000): Purine nucleoside phosphorylases: properties, functions, and clinical aspects *Pharmacol. Ther.* 88(3), 349-425
2. V. Gandhi *et al.* (1995): Cytotoxicity, metabolism, and mechanisms of action of 2',2'-difluorodeoxyguanosine in Chinese hamster ovary cells, *Cancer Res.* 55(7), 1517-1524
3. L. C. 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
4. C. U. Lambe *et al.* (1995): 6-Amino-6-methoxypurine arabinoside: an agent for T-cell malignancies, *Cancer Res.* 55(15), 3352-3356
5. M. Weibel (1994): Potentiating effect of {2[2(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl}ethenyl phosphonic acid (MDL 74,428), a potent inhibitor of purine nucleoside phosphorylase, on the antiretroviral activities of 2',3'-dideoxyinosine combined to ribavirin in mice, *Biochem. Pharmacol.* 48, 245-252
6. D. Shugar (1999): Viral and host-cell protein kinases: enticing antiviral targets, and relevance of nucleoside, and viral thymidine, kinases, *Pharmacol. Ther.* 82(2-3), 315-335

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 1970s¹ 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*². **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

	dCK assay	YMPK assay	Coupled dCK-YMPK assay	AK assay	5'cN-II assay
Natural substrates	Deoxyadenosine Deoxyguanosine Deoxycytidine Cytidine	dCMP, UMP CMP	Deoxycytidine Cytidine	Adenosine Inosine	Deoxyinosine Inosine
Nucleoside analogues substrates	Cladribine, fludarabine Gemcitabine, Lamivudine, Aracytidine Fluorodeoxyuridine	Monophosphate forms of cytidine analogues dFdCMP, araCMP, 3TCMP Adefovir (9-(2-phosphonomethoxyethyl) adenine	Gemcitabine Aracytidine Lamivudine Fluodeoxyuridine	Ribavirin Tubercidin Mizoribin	Dideoxyinosine Ribavirin Acyclovir

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

² Wu JZ, Larson G, Walker H, Shim JH, Hong Z. Phosphorylation of ribavirin and viramidine by adenosine kinase and cytosolic 5'-nucleotidase II: Implications for ribavirin metabolism in erythrocytes. (2005) *Antimicrob Agents Chemother*. 49(6):2164-71.

Human deoxycytidine kinase (dCK) Human, recombinant expressed in *E.coli* E.C. 2.7.1.74

Description

NOVO CIB'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.

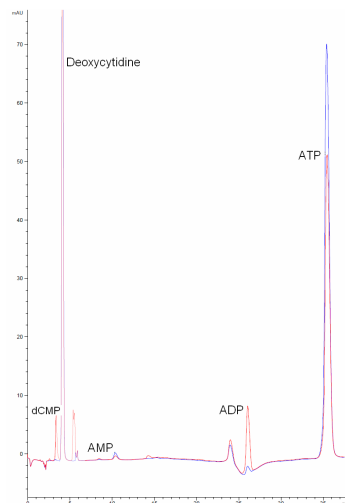
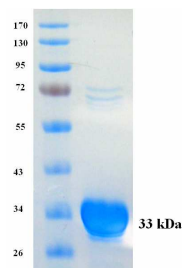
Deoxycytidine kinase is responsible for the phosphorylation and activation of numerous nucleoside analogs used to treat cancer (e.g. cytarabine, gemcitabine, cladribine and fludarabine) including nucleoside analogs of non-physiological L-chirality (e.g. 3TC, lamivudine, anti-HIV and anti-hepatitis B agent). Three-dimensional structures of dCK in complex with various pyrimidine¹ and purine^{2,3} 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.

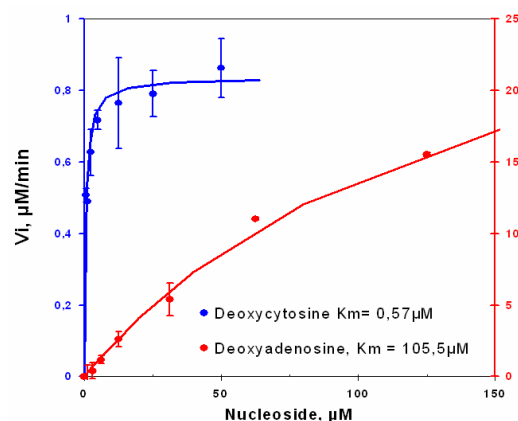
Unit Definition: One unit of deoxycytidine kinase converts 1.0 μ mole of deoxycytidine and ATP to dCMP and ADP per minute at pH 7.6 at 37°C, as measured by a coupled PK/LDH enzyme system.

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 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, 0.9 μ M 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:

NOVO CIB 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.

- **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 (cN-II)**
- **YMPK nucleotide monophosphate phosphorylation assay**
- **Adenosine kinase phosphorylation assay**
- **cN-II phosphorylation assay**

¹ Sabini E, Ort S, Monnerjahn C, Konrad M, Lavie A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. (2003) *Nat Struct Biol* 10(7):513-9

² Sabini E, Hazra S, Ort S, Konrad M, Lavie A. Structural basis for substrate promiscuity of dCK. (2008) *J Mol Biol* 378(3):607-21

³ Sabini E, Hazra S, Konrad M, Burley SK, Lavie A. (2007) Structural basis for activation of the therapeutic L-nucleoside analogs 3TC and troxacitabine by human deoxycytidine kinase. *Nucleic Acids Res* 35(1):186-92

dCK nucleoside phosphorylation assay

IMPORTANT: Client-specified alterations can be accommodated

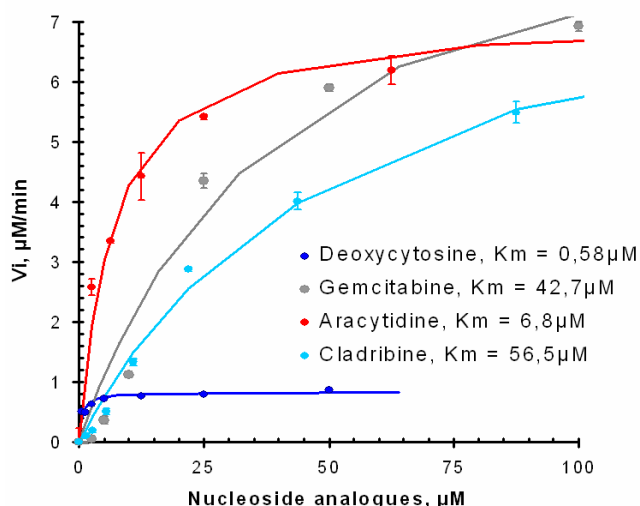
Aim: To characterize substrate properties (K_m and V_{max}) of new nucleoside analogues for human deoxycytidine kinase in comparison with those of known nucleoside analogues (e.g., **aracytidine**, **gemcitabine**, **cladribine** and **lamivudine**).

Substrate	Novocib*			Published data		Ref.
	$K_m, \mu M$	$V_{max}, \mu mol/mg/min$	Relative V_{max} , % of dCR	$K_m, \mu M$	$V_{max}, \mu mol/mg/min$	
Deoxycytidine	0,577	0,026	100	0,16	0,033	Recombinant Johansson Karlsson 1995
				1,3	0,069	Recombinant Usova & Eriksson, 1997
				0,57	0,004	Partially purified Someya H et al 2003
Gemcitabine	42,71	0,325	1250			
Deoxyadenosine	150,5	1,08	4153	115		Recombinant Sabini E et al 2008
				480	1,500	Recombinant Johansson Karlsson 1995
Aracytidine	6,81	0,224	862	15	0,009	Partially purified Someya H et al 2003
Cladribine	56,5	0,285	1096	89	0,126	Recombinant Usova & Eriksson, 1997
				24	0,76	Recombinant Johansson Karlsson 1995

Enzyme: The dCK used in the assays is a human recombinant dCK, cloned from human cells, expressed in *E. coli*, produced and purified by **NOVO CIB** (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, 0,9 μ M 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. K_m and V_{max} 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:

NOVO CIB 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.

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

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

Description

NOVO CIB's Human UMP-CMP kinase (YMPK) is a recombinant protein of ca. 27kDa (full length 228-aa form¹) 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 therapy². YMPK has a remarkable ability of to phosphorylate L-nucleotides from their monophosphate to diphosphate forms³ as shown for β -L-2'-3'-dideoxy-3-thiacytidine (L-SSdC, 3-TC or lamivudine), an anti-HIV and anti-hepatitis B drug.

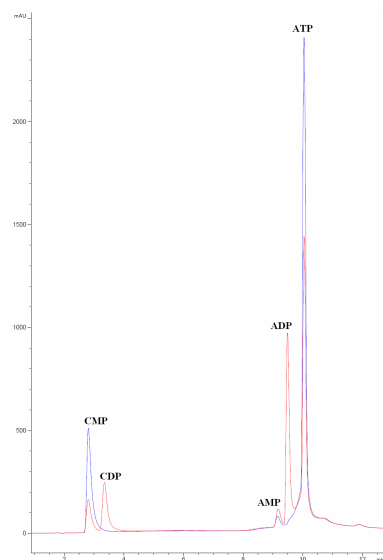
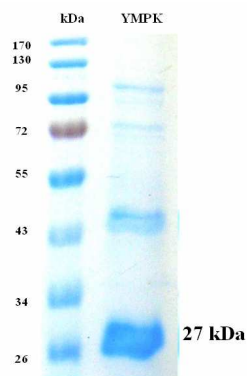
Crystal structure of open form of human UMP-CMP kinase has been solved recently⁴. These data together with homology model of enzyme in closed state provides structural basis for understanding the substrate specificity of the enzyme and helps to design of new nucleoside analogues of higher phosphorylation efficiency.

Storage: -20 °C in a solution containing 150mM KCl, 50mM Tris-HCl, pH7,5, 2mM β -mercaptotethanol, 50% glycerol.

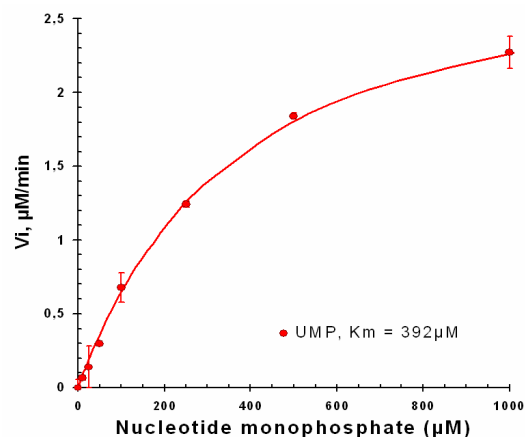
Unit Definition: One unit of UMP-CMP kinase converts 1.0 μ mole of UMP and ATP to UDP and ADP per minute at pH 7.6 at 25°C, using a coupled enzyme system with PK/LDH.

Specific activity: $\geq 0,150$ U/mg

Purity: controlled by SDS-PAGE



The enzymatic activity of recombinant UMP-CMP kinase was confirmed by HPLC analysis as illustrated by CDP and ADP formation (red) from CMP and ATP (blue).



Assay condition: Enzymatic activity of UMP-CMP kinase is measured by continuous spectrophotometric assays in a coupled lactate dehydrogenase/pyruvate kinase 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, 380nM YMPK. Reaction is followed in an iEMS Reader MF (Labsystems, Finland) microtiter plate reader at 340nm.

Related products:

NOVO CIB 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**

¹ Jieh-Yuan Liou, Ginger E. Dutschman, Wing Lam, Zaoli Jiang and Yung-Chi Cheng (March 2002) Characterization of Human UMP/CMP Kinase and Its Phosphorylation of D- and L-Form Deoxycytidine Analogue Monophosphates *Cancer Research* 62, 1624-1631

² Van Rompay AR, Johansson M, and Karlsson A (September 1999) Phosphorylation of Deoxycytidine Analog Monophosphates by UMP-CMP Kinase: Molecular Characterization of the Human Enzyme *Mol Pharmacol* 56 (3), 562-569

³ Claudia Pasti, Sarah Gallois-Montbrun, Hélène Munier-Lehmann, Michel Veron, Anne-Marie Gilles and Dominique Deville-Bonne (Mar2003) Reaction of human UMP-CMP kinase with natural and analog substrates. *European Journal of Biochemistry*, 270 (8), 1784 - 1790

⁴ Segura-Peña D, Sekulic N, Ort S, Konrad M, Lavie A. (2004) Substrate-induced conformational changes in human UMP/CMP kinase. *J Biol Chem*. 279(32):33882-33889

YMPK nucleotide monophosphate phosphorylation assay

IMPORTANT: Client-specified alterations can be accommodated.

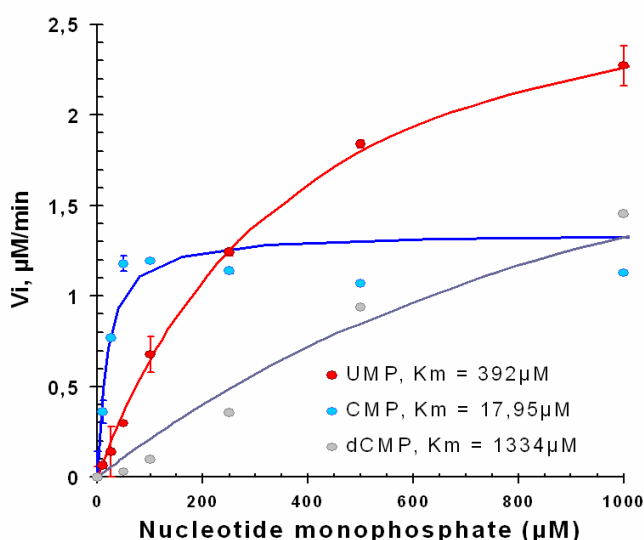
Aim: To characterize substrate properties (K_m and V_{max}) of monophosphate forms of new nucleoside analogues for human YMPK in comparison with monophosphate forms of natural nucleosides or reference nucleoside analogues.

Enzyme: The enzyme used in the assays is a human recombinant YMPK, cloned from human cells, expressed in *E. coli*, produced and purified by **NOVO CIB** (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).

			NovoCIB*	Published data ⁶	
	K_m , μM	V_{max} , nmol/mg/min	Relative V_{max} , % of CMP	K_m , μM	V_{max} , nmol/mg/min
CMP	17,9	130,07	100	20	350
UMP	392	307,16	236	45	350
dCMP	1334	297,65	228	900	200

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, 380nM 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. K_m and V_{max} 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:

NOVO CIB 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.

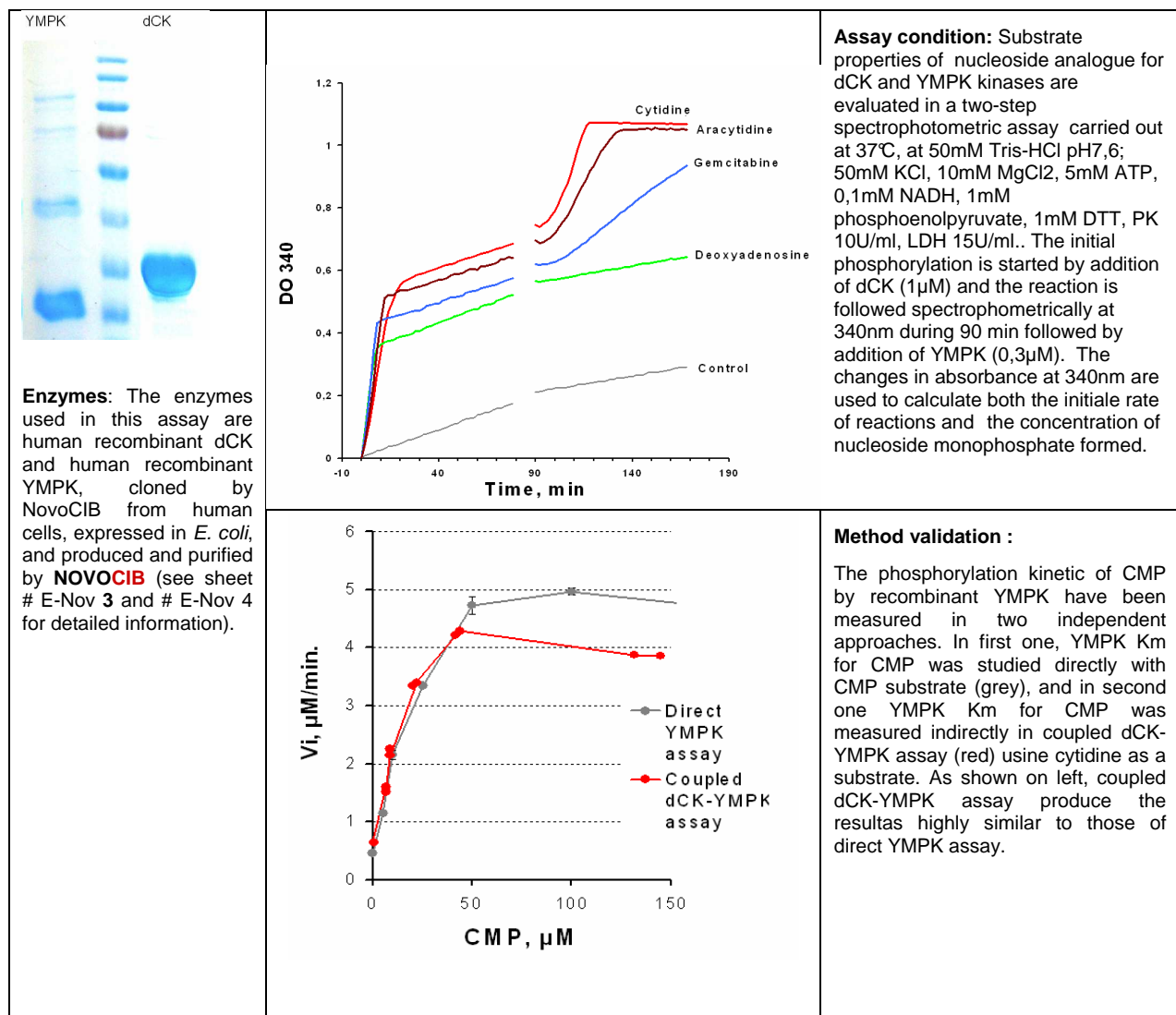
- **UMP-CMP kinase (YMPK)**
- **Coupled dCK-YMPK nucleoside phosphorylation assays**
- **Deoxycytidine kinase (dCK)**
- **Adenosine kinase (AK)**
- **Cytosolic 5' nucleotidase II (cN-II)**
- **dCK nucleoside phosphorylation assay**
- **Adenosine kinase phosphorylation assay**
- **cN-II phosphorylation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

References

Chih-Hung Hsu, Jieh-Yuan Liou, Ginger E. Dutschman, and Yung-Chi Cheng (2005) Phosphorylation of Cytidine, Deoxycytidine, and Their Analog Monophosphates by Human UMP/CMP Kinase Is Differentially Regulated by ATP and Magnesium *Mol Pharmacol* 67:806-814
Topalis D, Kumamoto H, Amaya Velasco MF, Dugué L, Haouz A, Alexandre JA, Gallois-Montbrun S, Alzari PM, Pochet S, Agrofoglio LA, Deville-Bonne D. (Jul 2007) Nucleotide binding to human UMP-CMP kinase using fluorescent derivatives – a screening based on affinity for the UMP-CMP binding site. *FEBS J.* 274(14):3704-14

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.



Related products:

NOVO CIB 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
- dCK nucleoside phosphorylation assay
- UMP-CMP kinase (YMPK)
- Deoxycytidine 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

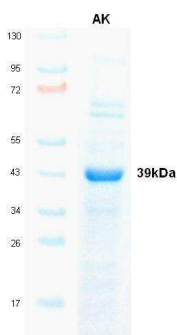
NOVO CIB's human adenosine kinase (AK) is a recombinant protein of ca.39kDa (345-aa short form^{1, 2}) cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in *E.coli*. The sequence of the cloned AK (GenBank accession number U50196) was confirmed by DNA sequencing (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 vivo*³. 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 epileptogenesis^{5, 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.

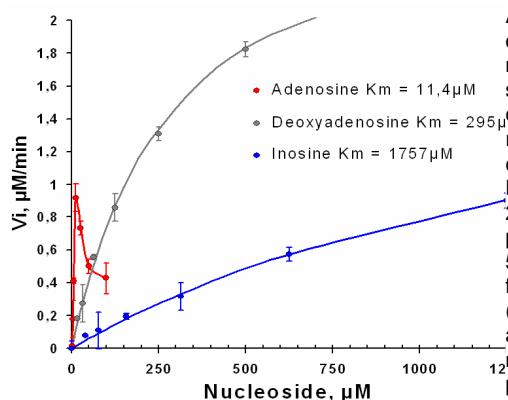
Storage: -20 °C in a solution containing 50 mM Tris-HCl, pH 7.6, 1 mM β -mercaptoethanol, 50% glycerol.

Unit Definition: One unit of adenosine kinase converts 1.0 μ mole 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.



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 pH7.6; 50mM KCl, 5mM MgCl₂, 2,5mM ATP, 0,1mM NADH, 1mM phosphoenolpyruvate, 1mM DTT, PK 5U/ml, LDH 5U/ml. 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:

NOVO CIB 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 phosphorylation assay
- 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

¹ . Spychala J, Datta NS, Takabayashi K, Datta M, Fox IH, Gribbins T, Mitchell BS Cloning of human adenosine kinase cDNA: Sequence similarity to microbial ribokinases and fructokinases (1996) *Proc. Natl. Acad. Sci. USA* 93, pp. 1232-1237

² Sahin B, Kansy JW, Nairn AC, Spychala J, Ealick SE, Fienberg AA, Greene RW and Bibb JA. Molecular characterization of recombinant mouse adenosine kinase and evaluation as a target for protein phosphorylation *Eur. J. Biochem.* 271, 3547–3555 (2004)

³ ABT-702 (4-Amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2,3-d]pyrimidine), a Novel Orally Effective Adenosine Kinase Inhibitor with Analgesic and Anti-Inflammatory Properties: I. In Vitro Characterization and Acute Antinociceptive Effects in the Mouse Jarvis MF, Yu H, Kohlhaas K, Alexander K, Lee CH, Jiang M, Bhagwat SS, Williams M, Kowaluk EA *Journal of Pharmacology and Experimental Therapeutics* 295:1156–1164, 2000

⁴ McGaraughty S, Cowart M, Jarvis MF, Berman RF. Anticonvulsant and antinociceptive actions of novel adenosine kinase inhibitors. *Curr Top Med Chem.* 2005;5(1):43-58

⁵ Fedele DE, Gouder N, Guttinger M, Gabernet L, Scheurer L, Rulicke T, Crestani F, Boison D Astroglial in epilepsy leads to overexpression of adenosine kinase, resulting in seizure aggravation (2005) *Brain*, 128, 2383–2395

⁶ Detlev Boison The adenosine kinase hypothesis of epileptogenesis *Progress in Neurobiology* 84 (2008) 249–262

⁷ Mathews, I.L., Erion, M.D. & Ealick, S.E. Structure of human adenosine kinase at 1.5 Å resolution. (1998) *Biochemistry* 37, 15607–15620

⁸ Willis RC, Carson DA, Seegmiller JE. Adenosine Kinase Initiates the Major Route of Ribavirin Activation in a Cultured Human Cell (1978) *PNAS* 75:3042-3044

⁹ Miller RL, Adamczyk DL, Miller WH, Koszalka GW, Rideout JL, Beacham LM, Chao EY, Haggerty JJ, Krenitsky TA, Elion, GB Adenosine kinase from rabbit liver II Substrate and inhibitor specificity. (1979) *J. Biol. Chem.* 254, 2346-2352

¹⁰ Saunders PP, Spindler CD, Tan MT, Alvarez E, Robins RK Tiazofurin Is Phosphorylated by Three Enzymes from Chinese Hamster Ovary Cells (1990) *Cancer Research* 50, 5269-5274

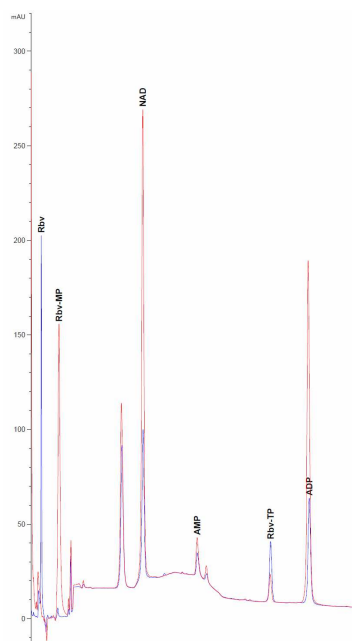
AK nucleoside phosphorylation assay

IMPORTANT: Client-specified alterations can be accommodated.

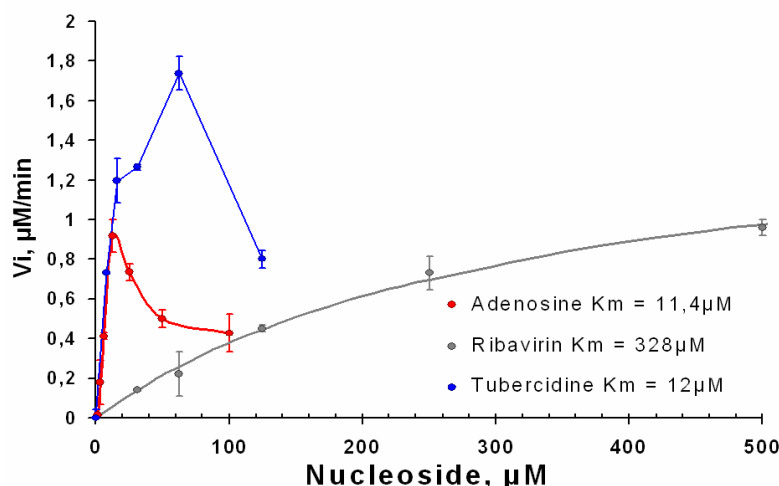
Aim: To characterize substrate properties (K_m and V_{max}) of new nucleoside analogues for human adenosine kinase in comparison with those of known nucleoside analogues (e.g., **ribavirine**, **tubercidine** or **mizoribine**).

Substrat	Novocib		Published		Ref
	K_m (μM)	K_{cat} (min^{-1})	K_m (μM)	K_{cat} (min^{-1})	
Adenosine	11	1.5	3.2	13	1
			0.150		2
Ribavirine	328	1,9	540	1,8	1
Deoxyadenosine	295	3,4	360		2
Tubercidine	12	2,2			
Inosine	1758	2,6			

Enzyme: The AK used in the assays is a human recombinant AK, cloned from human cells, expressed in *E. coli*, produced and purified by **NOVO CIB** (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).



The phosphorylation of ribavirin by adenosine kinase was confirmed by HPLC analysis as illustrated by Ribavirine-MP formation (red) from ribavirine (blue).



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. K_m and V_{max} 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:

NOVO CIB 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

1. Wu JZ, Larson G, Walker H, Shim JH, Hong Z. Phosphorylation of ribavirin and virmidine by adenosine kinase and cytosolic 5'-nucleotidase II: Implications for ribavirin metabolism in erythrocytes. (2005) *Antimicrob Agents Chemother.* 49(6):2164-71
2. Yamada, Y.; Goto, H.; Ogasawara, N. Adenosine kinase from human liver (1981) *Biochim. Biophys. Acta*, 660, 36-43

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

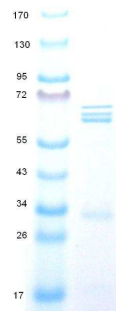
Description

NOVO CIB'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 nucleotidases¹ 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-bisphosphoglycerate⁴.

In addition, cytosolic 5'-nucleotidase II phosphorylates anti-viral and anti-tumour nucleoside analogues such as 2'3'-dideoxyinosine, carbovir⁵, acyclovir⁶ and ribavirin⁷.

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

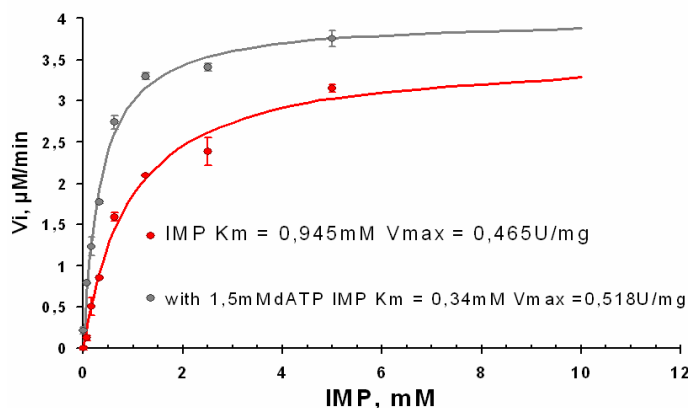


Unit Definition: One unit of 5'-nucleotidase converts 1.0 μmole of IMP to inosine per minute at pH 7.6 at 37°C, as measured by a coupled PNP/XO enzyme system in the presence of 20mM MgCl₂, 5mMDTT, 500μM KH₂PO₄, and 1,25mM IMP.

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 a 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 Mg Cl₂, 500μM KH₂PO₄, 5mM DTT, 119nM cN-II and various concentration of IMP. Reaction is followed at 295nm. The IMP is purchased from MPBiochemicals, XO is from Sigma-Aldrich and PNP is from Novocib (ref E-Nov-2).

Related products:

NOVO CIB 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.

- **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**

¹ Bianchi V, Spychala J. Mammalian 5'-nucleotidases (2003) *J. Biol Chem* 278(47): 46195-46198

² Allegrini S, Pesi R, Tozzi MG, Fiol CJ, Johnson RB, Eriksson S. Bovine cytosolic IMP/GMP-specific 5'-nucleotidase: cloning and expression of active enzyme in *Escherichia coli*. (1997) *Biochem J* 328:483-7.

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

⁴ Spychala J, Madrid-Marina V, Fox IH High Km Soluble 5'-nucleotidase from human placenta (1988) *J Biol Chem* 263(35): 18759-18765

⁵ Johnson MA, Fridland A. Phosphorylation of 2',3'-dideoxyinosine by cytosolic 5'-nucleotidase of human lymphoid cells. (1989) *Mol Pharmacol*. 36(2):291-5

⁶ Keller PM, McKee SA, Fyfe JA. Cytoplasmic 5'-nucleotidase catalyzes acyclovir phosphorylation (1985) *J Biol Chem*. 260(15):8664-7

⁷ Wu JZ, Larson G, Walker H, Shim JH, Hong Z. Phosphorylation of ribavirin and viramidine by adenosine kinase and cytosolic 5'-nucleotidase II: Implications for ribavirin metabolism in erythrocytes (2005) *Antimicrob Agents Chemother*. 49(6):2164-71

Coupled nucleoside kinase - IMPDH II assay

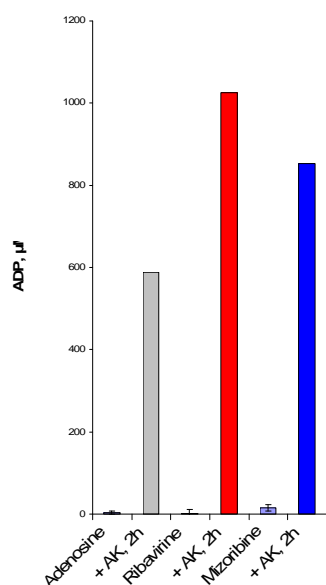
IMPORTANT: Client-specified alterations can be accommodated.

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.

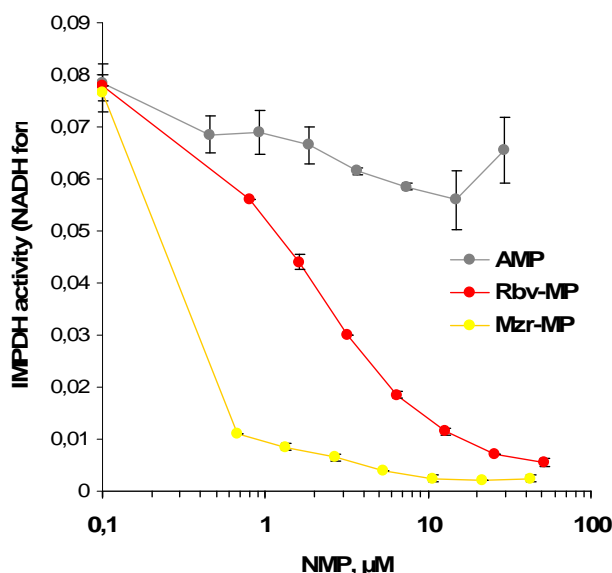
	dCK	AK	5'cN-II
Natural substrates	Deoxyadenosine Deoxyguanosine Deoxycytidine Cytidine	Adenosine Inosine	Deoxyinosine Inosine
Nucleoside analogues substrates	Cladribine Fludarabine Gemcitabine Lamivudine Aracytidine Fluorodeoxyuridine	Ribavirin Tubercidin Mizoribine	Dideoxyinosine Ribavirin Acyclovir

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 **NOVO CIB**. Human recombinant IMPDH 2 was cloned from human cells, expressed in *E. coli* and purified by **NOVO CIB** (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.



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

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



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.

References

- [1] P. Leyssen, J. Balzarini, E. De Clercq, J. Neyts (2005) The Predominant Mechanism by Which Ribavirin Exerts Its Antiviral Activity In Vitro against Flaviviruses and Paramyxoviruses Is Mediated by Inhibition of IMP Dehydrogenase J Virol 79: 1943–1947
- [2] L.J. Stuyver, S. Lostia, S.E. Patterson, J.L. Clark, K. A. Watanabe, M.J. Otto and K.W. Pankiewicz (2002) Inhibitors of the IMPDH enzyme as potential antiviral agents viral diarrhoea virus agents Antiviral Chemistry & Chemotherapy 13:345–352

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.

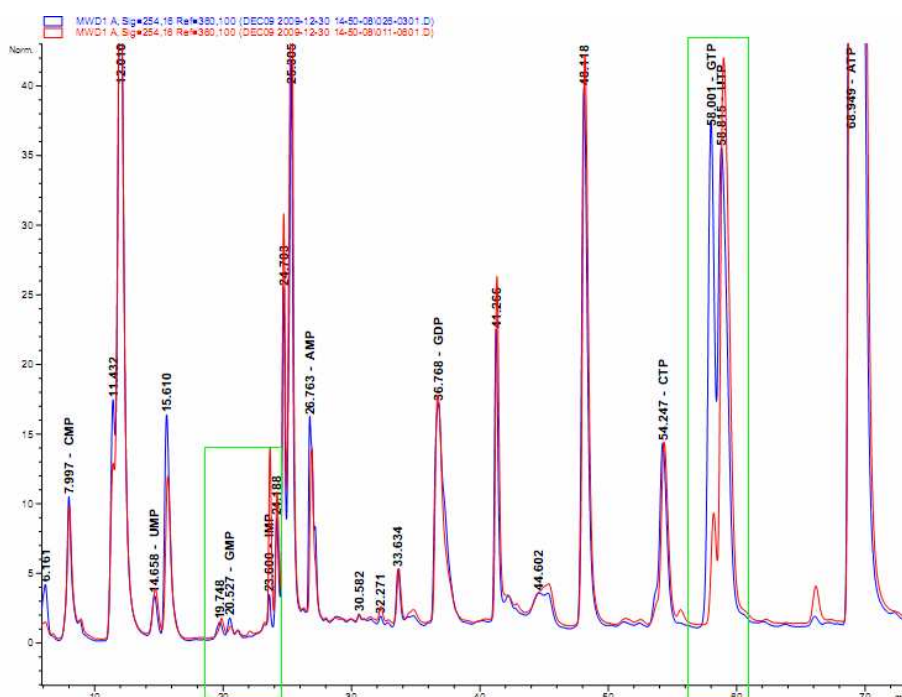
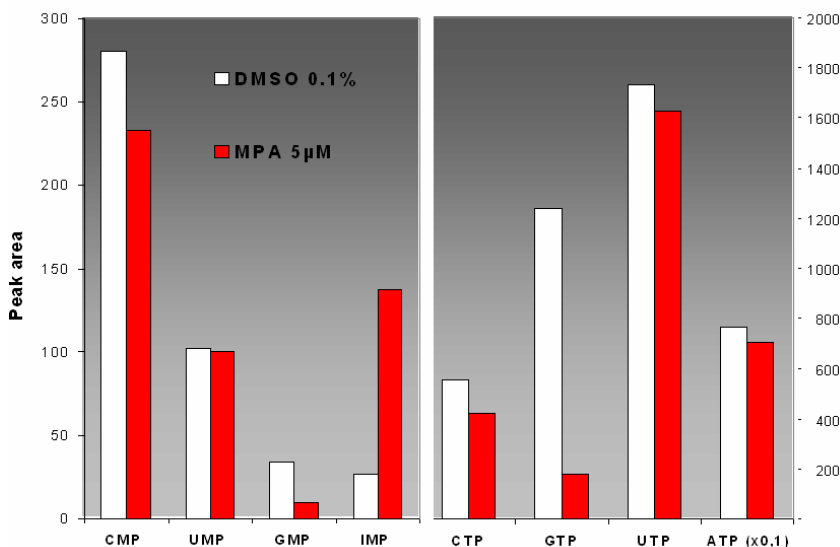


Figure 1. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.



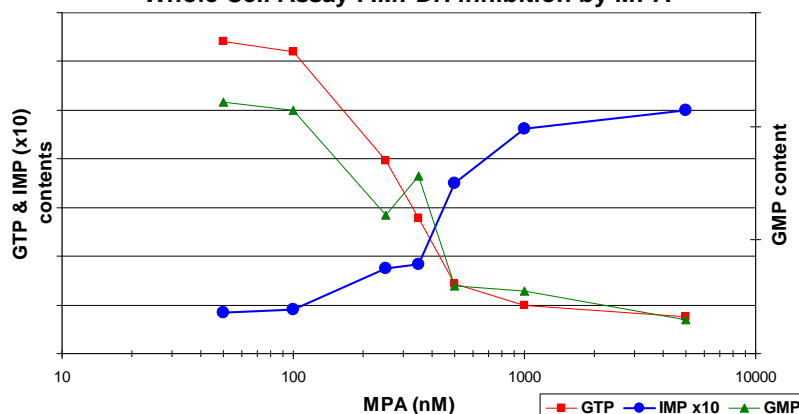
Concentration of nucleotides mono- and tri-phosphate in MPA- and DMSO-treated cells (measures as peak area, AU)

	DMSO 0.125%	MPA 5µM
CMP	280.2	233.2
UMP	102.2	100.2
GMP	33.8	9.6
IMP	27.0	137.2
AMP	396.6	273.2
CTP	554.0	424.8
GTP	1,237.0	182.0
UTP	1,734.8	1,627.0
ATP	7,665.0	7,057.0

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)

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.

Whole Cell Assay : IMPDH inhibition by MPA



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.

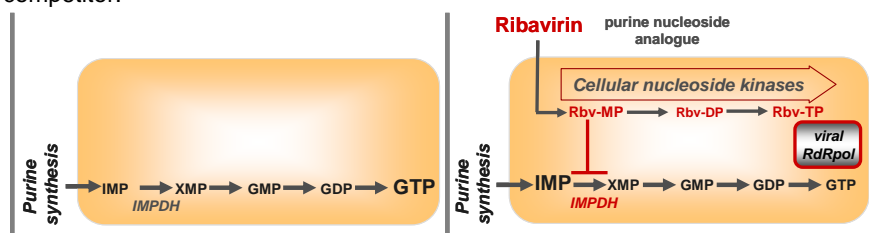


Figure 3. Modifications in cell-pool of nucleotides in Ribavirin-treated cells

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-paired chromatography and quantified. This cellular assay was validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, 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.

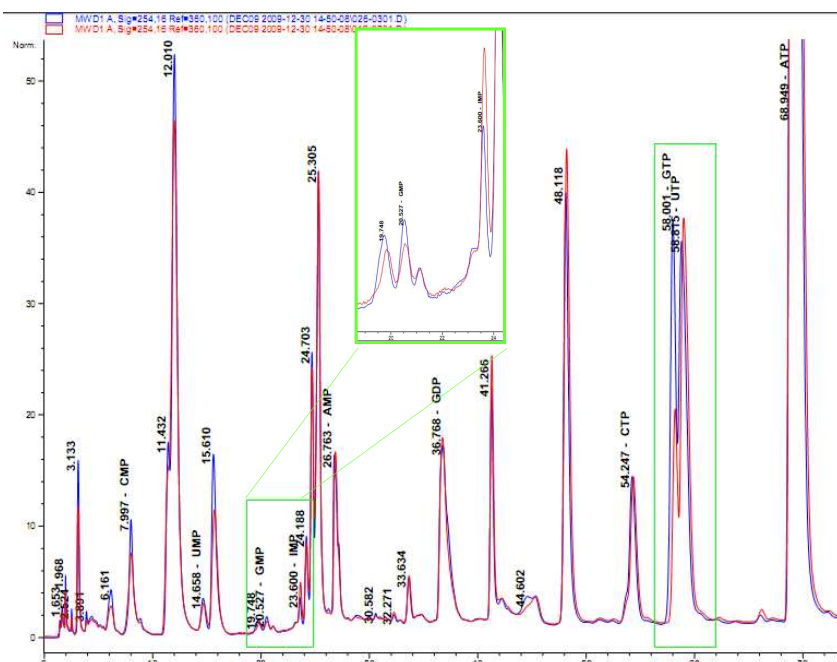
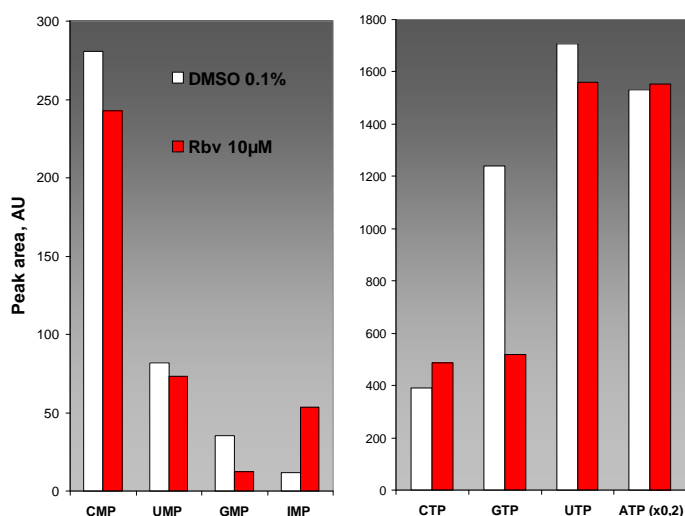


Figure 4. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 10µM Rbv (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.



Concentration of nucleotides mono- and tri-phosphate in Rbv- and DMSO-treated cells (measures as peak area, AU)

	DMSO 0.125%	Rbv 10µM
CMP	281.0	242.8
UMP	81.8	73.2
GMP	35.1	12.5
IMP	11.6	53.4
AMP	335.0	341.6
CTP	392.3	488.0
GTP	1,238.0	519.4
UTP	1,708.0	1,561.0
ATP	7,658.0	7,766.0

Figure 5. Effects of 10µM Rbv on cellular pool of nucleotide mono- and di-phosphates (results of quantification of HPLC spectra presented on Figure 4)

Materials & Methods

Cells treatment

Huh-7 cells are grown in an atmosphere of humidified 5% CO₂ at 37°C in DMEM medium supplemented with 2mM L-glu tamine, 10% heat-inactivated fetal bovine serum and streptomycin-penicillin. Exponentially grown Huh-7 cells are seeded at ~6x10⁵ 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% acetonitril for 1h. The extracts are centrifuged to remove cellular debris and nucleotides are extracted by SPE procedure (SAX column, Supelco, Sigma-Aldrich) pre-conditioned 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 nucleotides¹ 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).

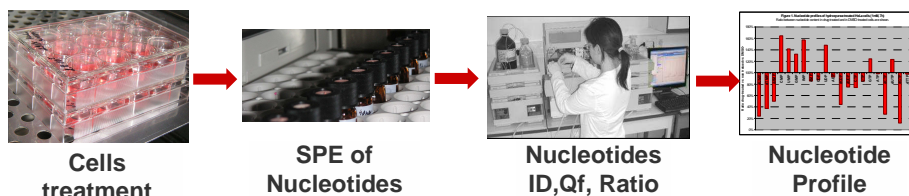
¹ D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio', B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407-412

RNR inhibition - Whole Cell Assay

IMPORTANT: Client-specified alterations can be accommodated.

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)

Nucleotide profiles of hydroxyurea-treated HeLa cells (1mM, 20h)

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

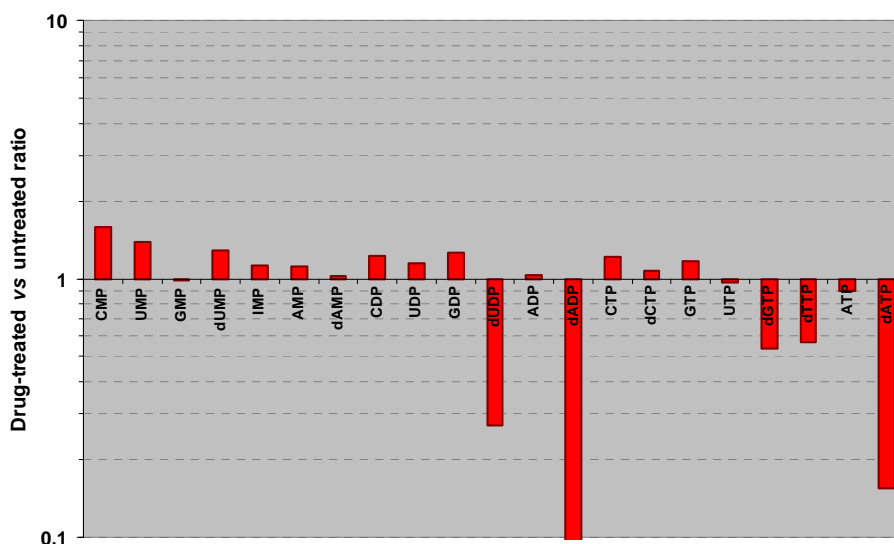


Figure 1: Nucleotide profiles of hydroxyurea-treated HeLa cells

Hydroxyurea is an antineoplastic agent, antimetabolite, 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^{1, 2}. 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^{1, 2}.

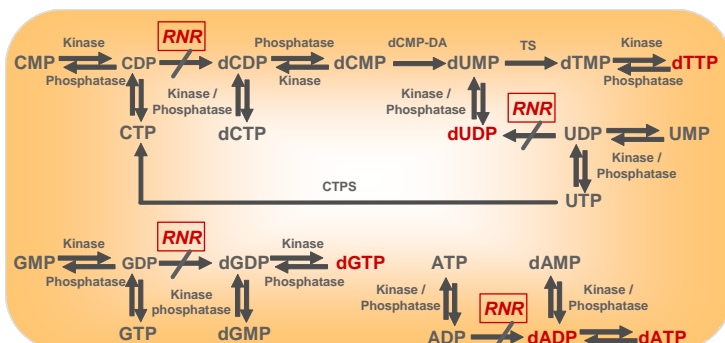


Figure 2. Effect of hydroxyurea on cellular pool of deoxynucleotides. The depleted nucleotides are shown in red. Ribonucleoside reductase (RNR), a recognized target of hydroxyurea, is framed in red.

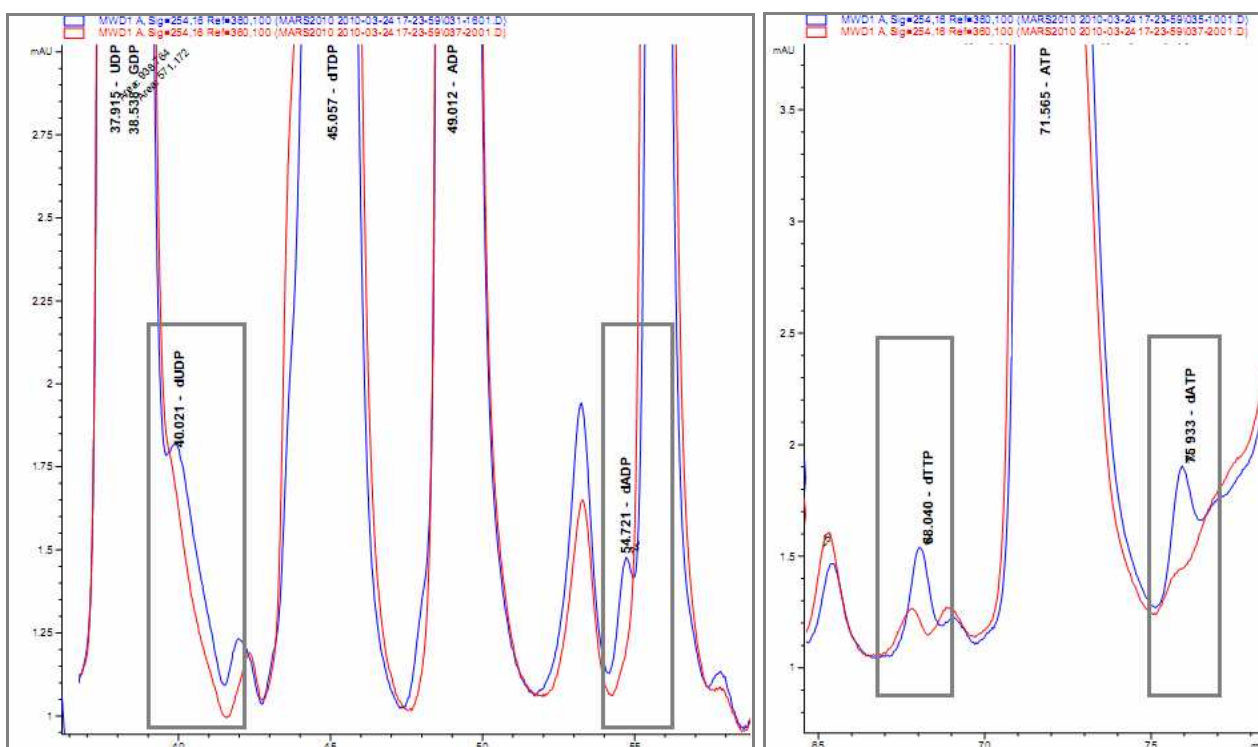


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and i dTTP, dATP on right.

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.

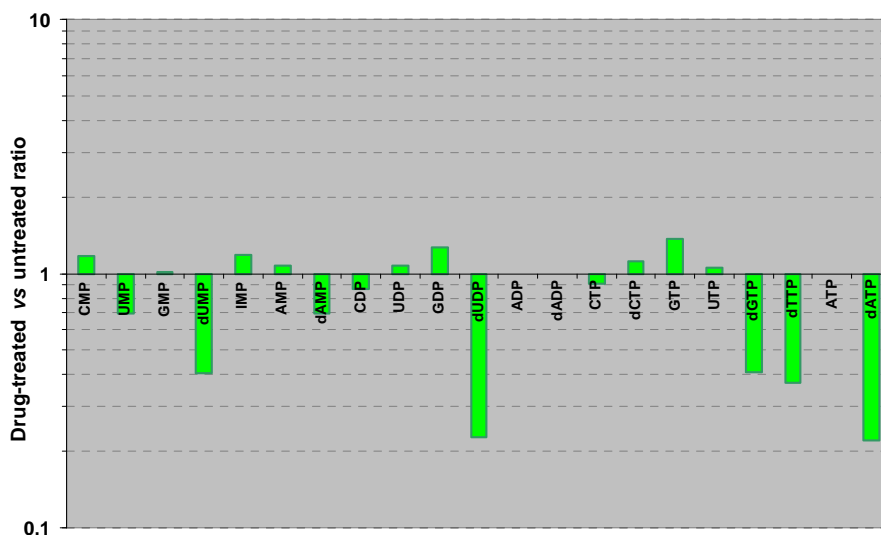


Figure 4: Nucleotide profiles of gemcitabine-treated HeLa cells

¹ Bianchi, V., Pontis, E., and Reichard, P. (1986) **Changes of Deoxyribonucleoside Triphosphate Pools Induced by Hydroxyurea and Their Relation to DNA Synthesis** *J. Biol. Chem.* 261, 16037–16042

² S. P. Hendricks and C. K. Mathews (1998) **Differential Effects of Hydroxyurea upon Deoxyribonucleoside Triphosphate Pools, Analyzed with Vaccinia Virus Ribonucleotide Reductase.** *J. Biol. Chem.* 273, 29519–523

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 data¹, but may also reflect the decrease in cellular dUDP, source of dUMP.

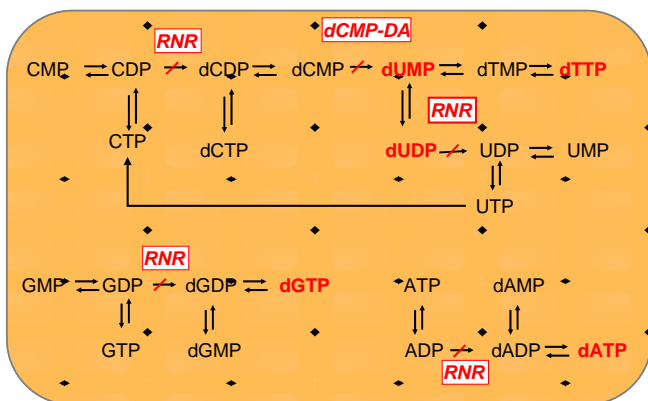


Figure 5. Effect of gemcitabine on cellular pool of nucleotides and deoxynucleotides. The depleted (<50% of control) nucleotides are shown in red. Ribonucleotide reductase (RNR) and dCMP-deaminase (dCMP-DA), recognized targets of gemcitabine are framed in red.

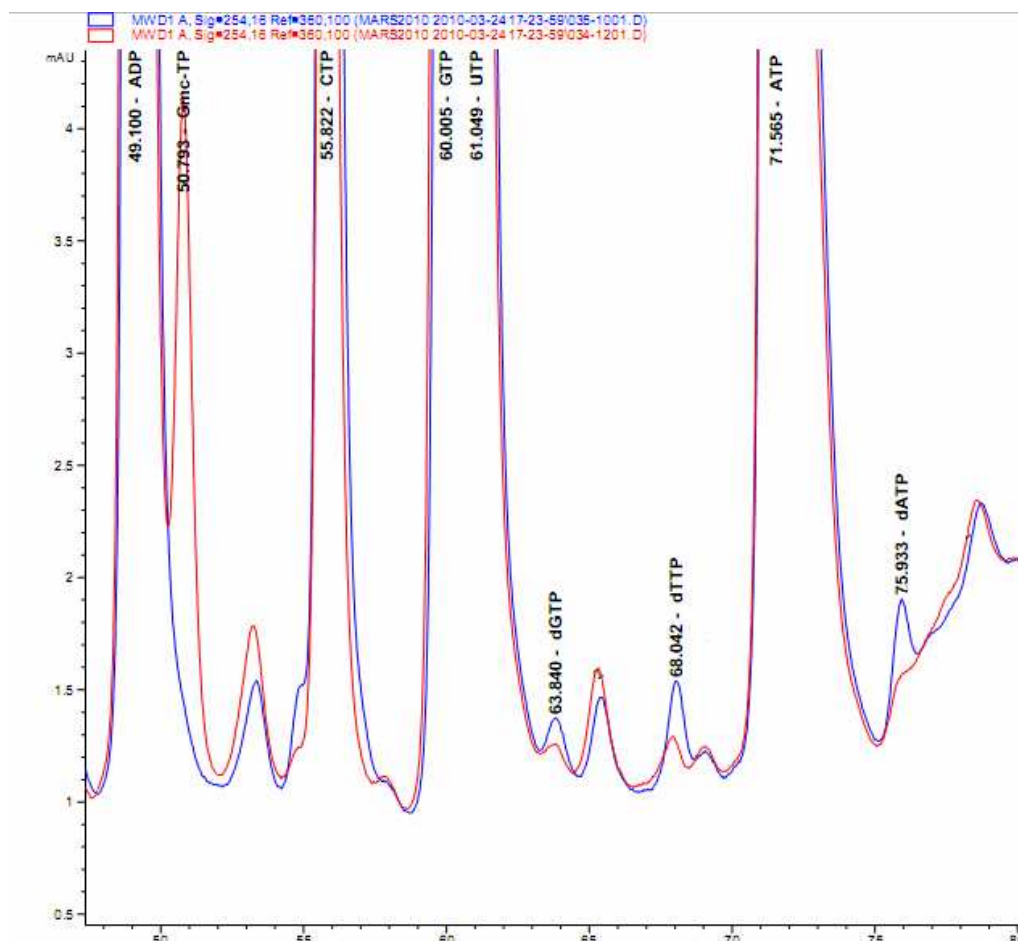


Figure 6. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 37 μ M gemcitabine (blue) and DMSO (red) illustrating depletion in dNTP.

¹ C. J.A. van Moorsel, A. M. Bergman, G. Veerman, D. A. Voorn, V. W.T. Ruiz van Haperen, J. R. Kroep, H. M. Pinedo, G. J. Peters (2000) **Differential effects of gemcitabine on ribonucleotide pools of twenty-one solid tumour and leukaemia cell lines** *Biochimica et Biophysica Acta* 1474, 5-12

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 DMSO 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 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 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, 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%.

¹ D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio¹, B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407–412

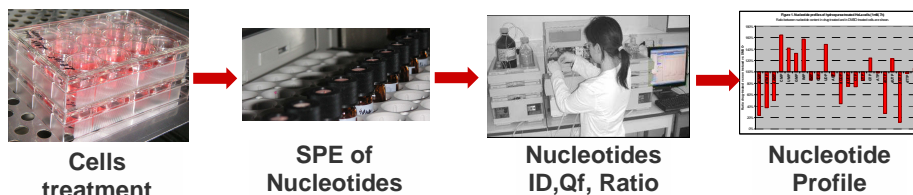
Multi-targeted Antifolates - Whole Cell Assay

Inhibition of *de novo* purine and pyrimidine biosynthesis

IMPORTANT: Client-specified alterations can be accommodated.

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.



Nucleotide profiles of methotrexate-treated HeLa cells (10µM, 20h)

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

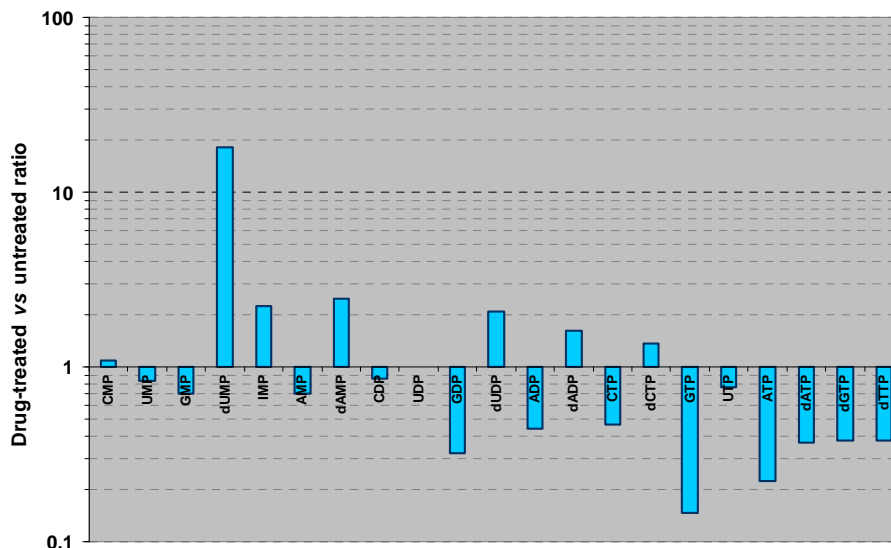


Figure 1: Nucleotide profiles of methotrexate-treated HeLa cells

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 arthritis¹. MTX was first believed to be an inhibitor of the enzyme dihydrofolate reductase (DHFR), the enzyme required for reduction of dihydrofolate (FH₂) to tetrahydrofolate (FH₄). However, as shown in the 80's, MTX is actually a prodrug which is polyglutamated and accumulated in cells². 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)³ and the thymidylate synthetase (TS)⁴.

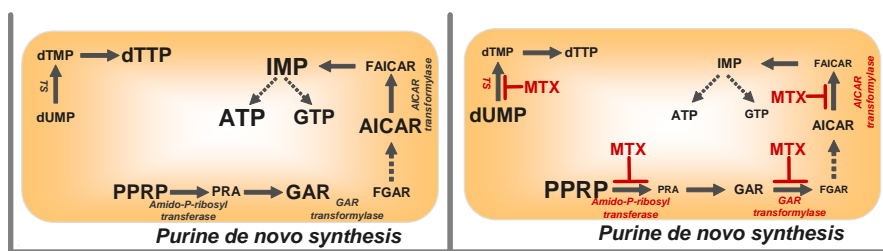


Figure 2. Effect of MTX on cellular pool of purines and pyrimidines.

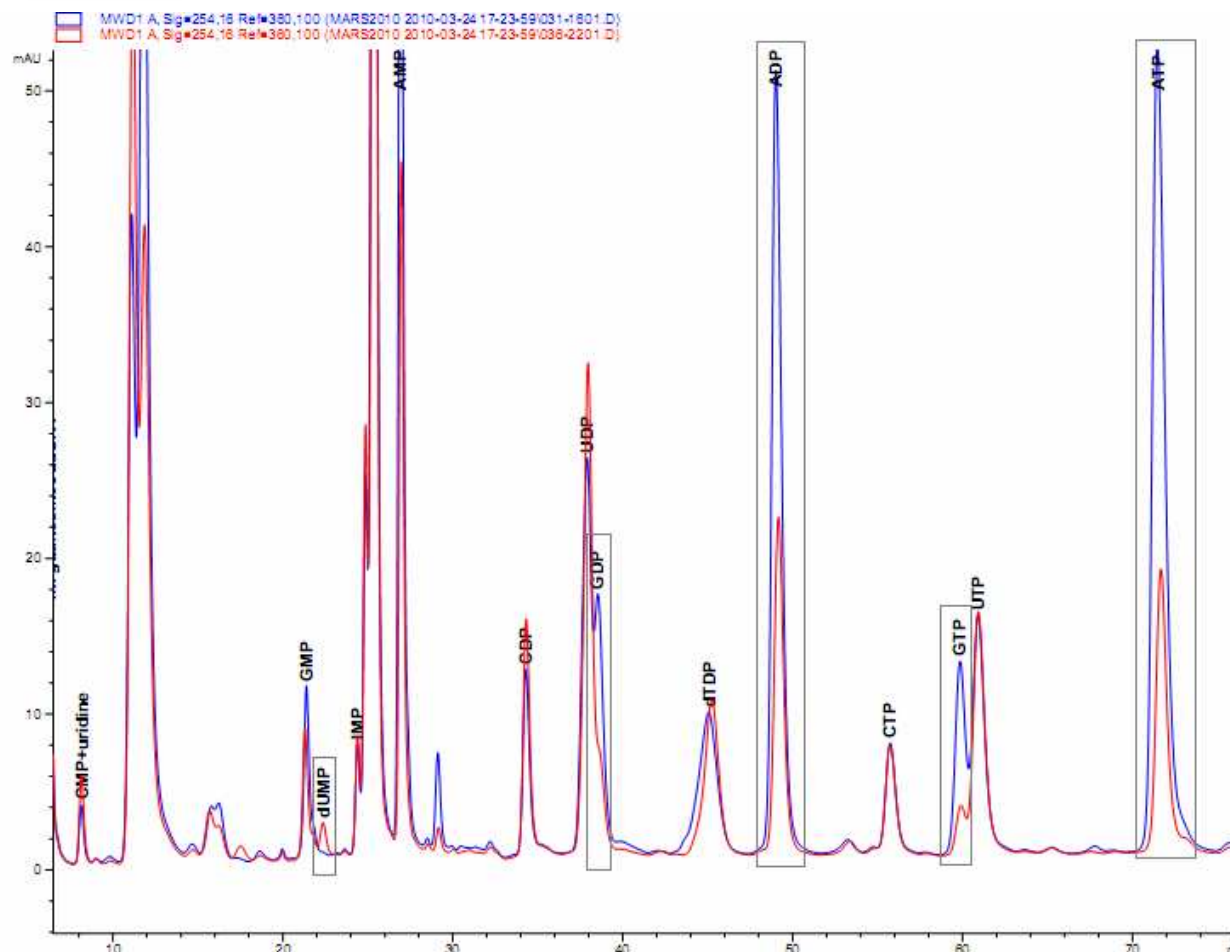


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 10µM MTX (red) and DMSO (blue). The specific depletion in purine nucleotides, e.g. ATP, ADP, GTP and GDP, and accumulation of dUMP, as a result of TS inhibition, are framed in grey.

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 data⁵ showing that MTX inhibits *de novo* synthesis of purine nucleotides through AICART enzyme and synthesis of thymidylate through thymidylate synthase.

¹ L.D. Fairbanks, K. Ruckemann, Y. Qiu, C.M. Hawrylowicz, D.F. Richards, R. Swaminathan, B. KIRSCHBAUM and H.A. Simmonds (1999) **Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes: a metabolic basis for efficacy in rheumatoid arthritis?** *Biochem. J.* 342, 143-152

² B.A. Chabner, C.J. Allegra, G.A. Curt, N.J. Clendeninn, J. Baram, S. Koizumi, J.C. Drake, and J. Jolivet (1985) **Polyglutamation of Methotrexate- Is Methotrexate a Prodrug?** *J. Clin. Investig.* 76, 907-912

³ C.J. Allegra, K. Hoang, G.C. Yeh, J.C. Drake and J. Baram (1987) **Evidence for direct inhibition of de novo purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate.** *J. Biol. Chem.* 262, 13520-13526.

⁴ C J Allegra, B A Chabner, J C Drake, R Lutz, D Rodbard and J Jolivet (1985) **Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates** *J. Biol. Chem.* 260, 9720-9726.

⁵ G.P. Budzik, L.M. Colletti and C.R. Faltynek (2000) **Effects of methotrexate on nucleotide pools in normal human cells and the CEM T cell line** *Life Sciences.* 66(23), 2297-2307

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 DMSO 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.

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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 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 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, 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%.

¹ D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio', B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407–412

Nucleoside & Nucleotide Analysis by HPLC

ATP/ADP ratio, NAD pool... or whole metabolic profiles

IMPORTANT: Client-specified alterations can be accommodated.

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:

ATP	ADP	AMP	NAD	NADP
GTP	GDP	GMP	IMP	
UTP	UDP	UDP-glucose	UMP	CTP
NADH	NADPH	dATP	dGTP	dTTP
Guanine	Uracyl	Hypoxanthine	Inosine	Uridine
Cytosine	Cytidine			

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 $\sim 10^7$ treated cells, per compound and per concentration tested. Control untreated cells are cultured under the same conditions to provide a reference metabolic profile.

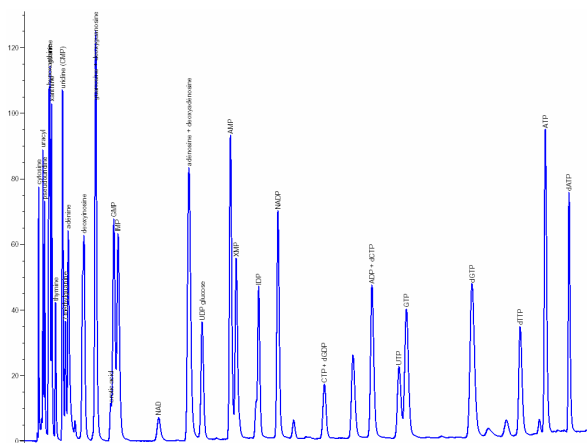
Depending on the cell line or the experimental conditions, a $0.5-1.10^5$ cell-extract can be sufficient to analyze the major metabolites (e.g. ATP, ADP...).

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)

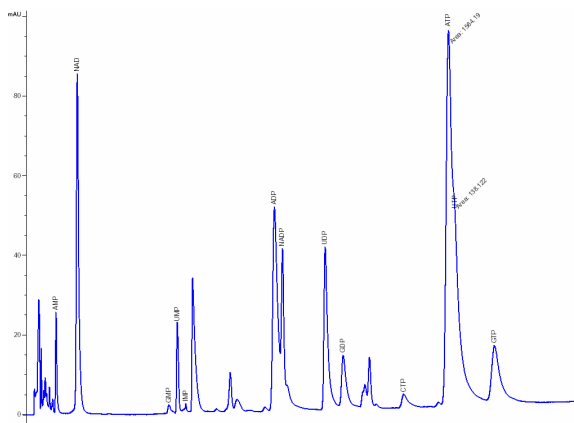
If needed, for instance to focus on naturally low-level metabolites, larger samples can be prepared, e.g. up to 10^8 of cultured cells

Nucleosides & nucleotides separation and analysis: The extraction and separation procedures have been optimized and specially developed by **NOVO CIB**. 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

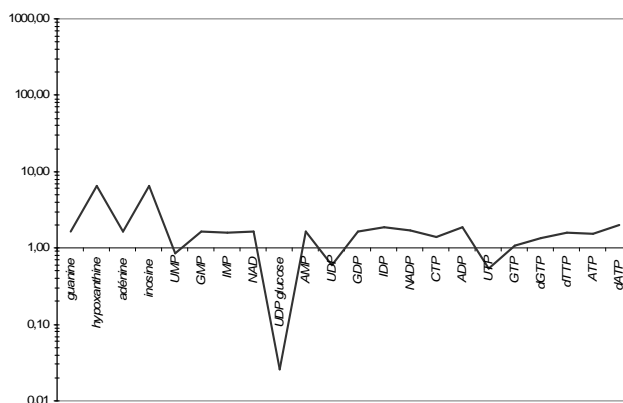
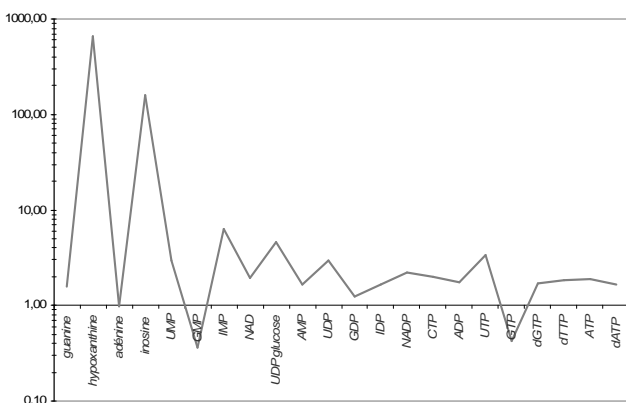


Metabolites	pmol / 10 ⁶ cells *	published data **
UMP	728.1 +/- 56,8	
GMP	34.4 +/- 4,8	
IMP	72.7 +/- 21,4	(130)
NAD	1 825.1 +/- 155,5	
UDPGlu	1 113.9 +/- 87,1	
AMP	122.7 +/- 12,9	
UDP	1 730.3 +/- 207,1	
CTP	2 754.5 +/- 165,4	
ADP	4 652.4 +/- 609,8	
UTP	2 494.2 +/- 156,1	
GTP	1 449.7 +/- 81,4	(1540)
ATP	6 561.0 +/- 346,0	(6580)

* Mean +/- SD for 5 independent experiments

** J. Balzarini *et al.* (1993): **Eicar (5-ethynyl-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 **NOVO CIB** 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 **NOVO CIB**, we routinely measure ribavirin and ribavirin 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.

Cellular Pharmacology of Nucleoside Analogues

IMPORTANT: Client-specified alterations can be accommodated.

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...

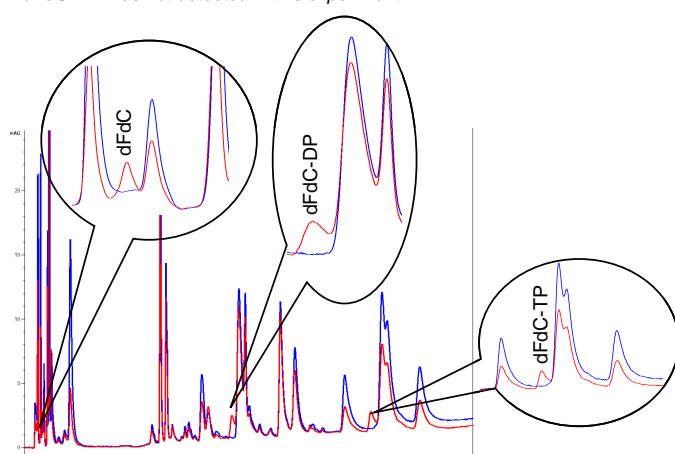
Do not hesitate to contact us for any matter of feasibility!

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 μ M

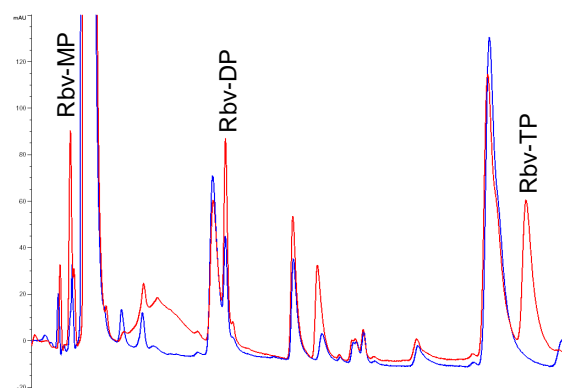
* dFdC-MP was not detected in this experiment



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

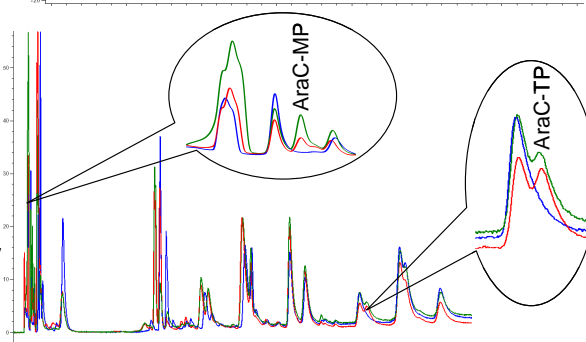


Separation of cytarabine (AraC) and cytarabine mono- (AraC-MP), di- (AraC-DP) and triphosphate (AraC-TP) in Huh7 cell extract:

Blue: non-treated cells

Red: 48h-treatment with cytarabine (AraC) at 130 μ M

Green: 48h-treatment with cytarabine (AraC) at 260 μ M



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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_i 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.

NOVO CIB 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

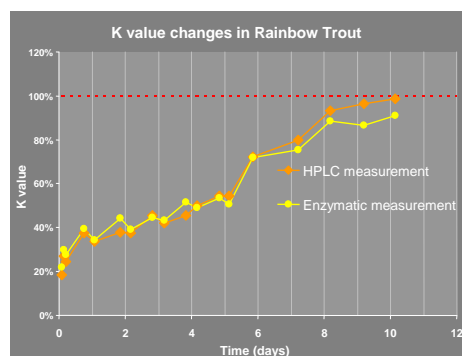
About 1g of muscle (preferably dorsal muscle for finfish) is collected and heated in boiling water for 2-4 minutes.

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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Baby Milk Nucleotide Analysis

HPLC analysis of NMP's in baby milk powder

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. **NOVO CIB** 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

Nucleotide	R ²	LOD (mg /100g)
CMP	0,9989	0,025
AMP	0,9990	0,025
IMP	0,9992	0,050
UMP	0,9987	0,025
GMP	0,9990	0,025

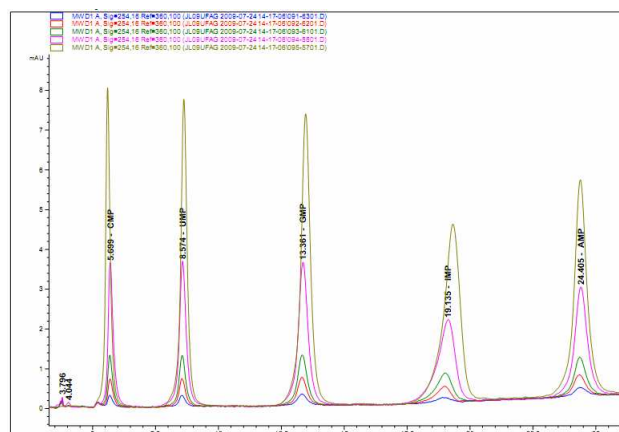


Fig. 1. Superposed spectra of 5 concentrations of standards (0.25, 0.5, 1.25, 2.5 and 5mg/100g).

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)

	Mean	SD
CMP	77%	14%
UMP	94%	16%
GMP	89%	19%
IMP	84%	9%
AMP	97%	19%

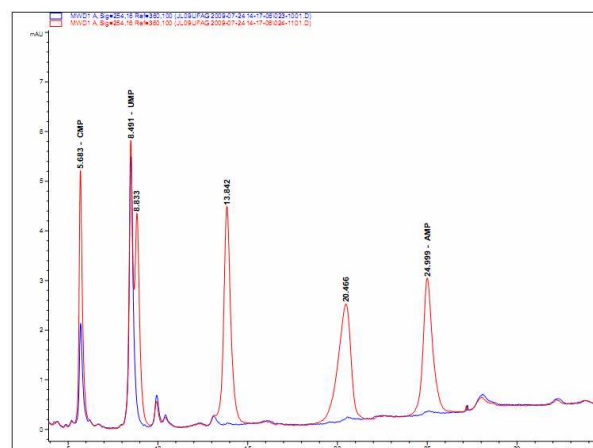


Fig. 2. Superposed chromatograms of milk powder without exogenous nucleotide (blue) and of a milk powder spiked with NMP at 2.5mg/100g (red).

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