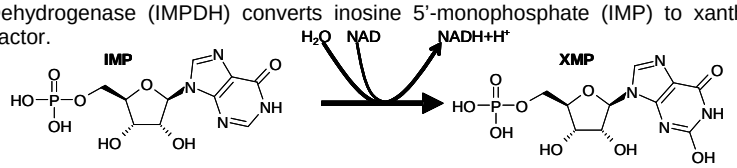
IMPDH II, a choice target for major therapeutic applications

Catalytic activity

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



The oxidation of IMP to XMP is considered as the pivotal step in the biosynthesis of guanine nucleotide, whose pool controls cell proliferation and many other major cellular processes[1]. The decrease in guanine nucleotide resulting from IMPDH inhibition interrupts the nucleic acid synthesis in proliferating cells. The involvement of IMPDH in *de novo* guanine nucleotide biosynthesis makes IMPDH a crucial enzyme in cell proliferation and differentiation[2]. IMPDH is recognized as a validated target for several major therapeutic areas. IMPDH inhibitors are exploited as antiviral (*e.g.* ribavirine), antiparasitic, antimicrobial, antileukemic and immunosuppressive agents[2]. IMPDH Type II is the predominant isoform of the enzyme and is selectively expressed in proliferating cells, including lymphocytes and tumor cells[2].

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].

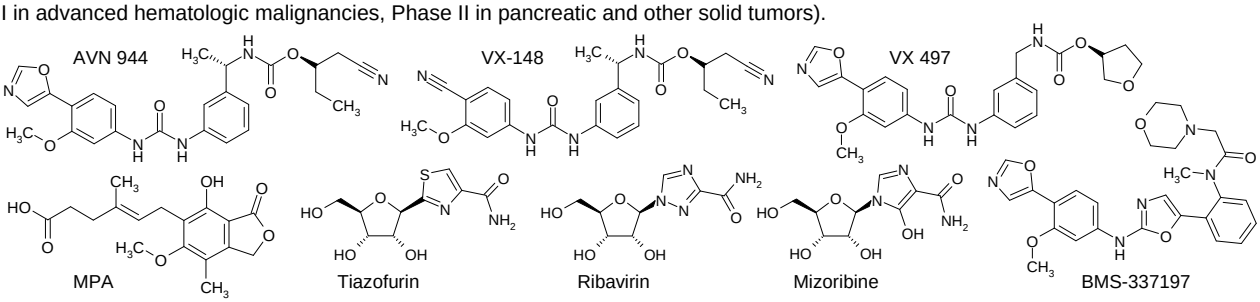
IMPDH in oncology

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 resistance[7] that may appear in certain tumors. For instance, methotrexate resistance is directly related to the overexpression of IMPDH, whose inhibition restores the drug efficacy[8]. Combination with other anti-cancer drugs extends the potential application of IMPDH inhibitors.

Current development of IMPDH inhibitors

CellCept®, ribavirin, mizoribine and tiazofurine are examples of currently used drugs that target IMPDH. Benzamide riboside, tiazofurine, MPA are under development in Phase II/III in leukemia: results are judged very encouraging[8].

The IMPDH II atomic structure has been resolved and it provides a valuable background for further leads optimization[9]. 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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4. L. Chen and K. W. Pankiewicz (2007): **Recent development of IMP dehydrogenase inhibitors for the treatment of cancer** *Curr Opin Drug Discov Devel.* 10(4):403-12 (Review)
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6. K. Malek *et al.* (2004): **Effects of the IMP-dehydrogenase inhibitor, Tiazofurin, in bcr-abl positive acute myelogenous leukemia** *Leukemia Research* 28, 1125–1136
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8. S. Peñuelas *et al.* (2005): **Modulation of IMPDH2, survivin, topoisomerase I and vimentin increases sensitivity to methotrexate in HT29 human colon cancer cells** FEBS 272, 696–710
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12. J. Jain *et al.* (2004): **Regulation of inosine monophosphate dehydrogenase type I and type II isoforms in human lymphocytes** *Biochem. Pharmacol.* 67(4), 767-776
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Inosine Monophosphate Dehydrogenase Type II (IMPDH II)

Human, recombinant expressed in E. coli

E.C. 1.1.1.205

Synonyms: inosine 5'-monophosphate dehydrogenase, type 2 IMP dehydrogenase type II, IMPDH2

Description

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

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

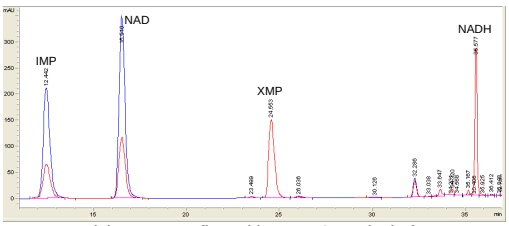
IMPDH is involved in *de novo* guanine nucleotide biosynthesis. It plays a major role in cell growth and in the malignancy of some tumors. Additionally, guanine nucleotide is needed for lymphocyte proliferation.
IMPDH II is the predominant isoform of IMPDH. It is recognized as a validated target to treat a wide range of cancers and infectious diseases and to prevent lymphocytes proliferation (for further details, see "IMPDH II, a choice target for major therapeutic applications").

Storage: –70 ℃ in a solution containing 50 mM KH_2PO_4, 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 ℃

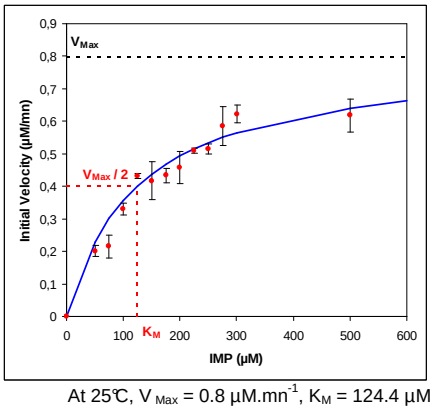
Specific Activity: ≥ 0.035 unit/mg protein.

Purity controlled by SDS-PAGE

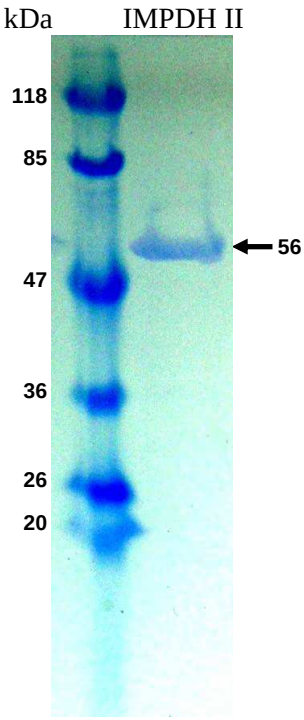


IMPDH activity was confirmed by HPLC analysis for quantification of IMP, XMP, NAD and NADH

Assay condition: KH_2PO_4 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℃. Reaction started by adding IMP at various concentrations. NADH formation was measured in an iEMS Reader MF (Labsystems, Finland) microtiter plate reader at 340nm.



At 25℃, V_{Max} = 0.8 µM.mn^{-1}, K_M = 124.4 µM



IMPDH inhibition assays

NOVOCIB 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.

***In vitro* Assay**
for Screening & Kinetic Analysis (IC_{50})
- **with Human Recombinant IMPDH II**
- **with Bacterial (*Staphylococcus aureus*) IMPDH**

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

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

IMPDH II - *In vitro* Assay

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 IC_{50} value.

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

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

Enzyme concentration: Bradford method

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

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℃ or 37℃ in 200µl of reaction buffer on 96-well microplate.
Reaction buffer is: KH_2PO_4 0.1M, pH7.8, NAD 180µM, 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 50µM
- For Kinetics Analysis (IC_{50}): 11 positive controls containing MPA as an IMPDH inhibitor, at 11 concentrations which are equally spaced by 3-fold dilutions to cover a 4.8-log wide range, as follows:

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

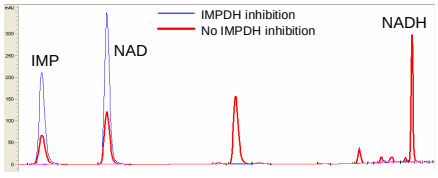
Controls are done in duplicate. If an additional microplate is needed, it includes the complete set of controls (in duplicate). Additional concentrations of inhibitor can be tested.

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 100µM (final concentration)

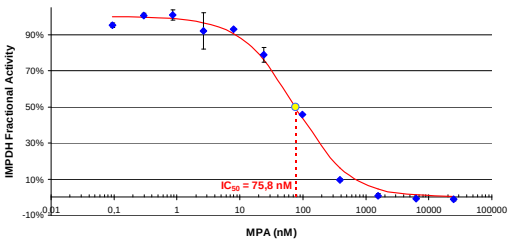
*Solubility in the reaction buffer must be checked before performing the assay.

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: $\Delta A / \varepsilon_{NADH} . p . t$ 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 200µl/well), *t* is the maximal time ($t \leq 30$mn) at which velocity (NADH formation rate) remains constant.

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



IMPDH II inhibition by MPA at 25℃



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

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

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

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

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

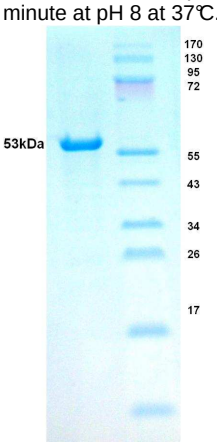
Description
NOVOCIB's bacterial IMPDH is a recombinant protein of *ca. 53*kDa cloned by PCR amplification of *guaB* gene of *Staphylococcus aureus* and expressed in *E.coli*.

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

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

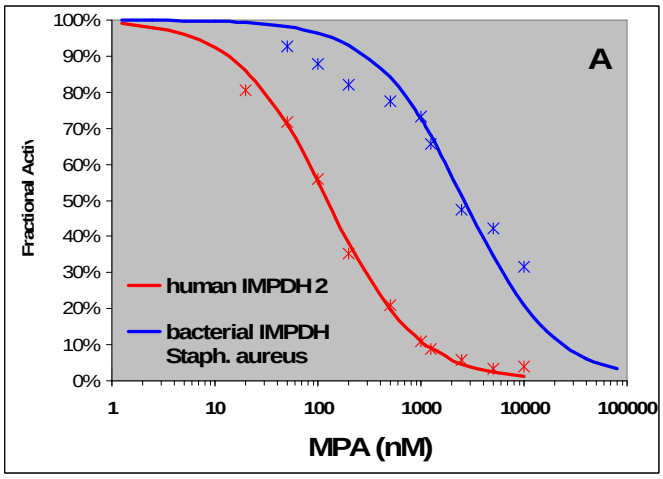
Both bacterial recombinant IMPDH and human recombinant IMPDH are available from **NOVOCIB** providing the tools for selection of species-specific IMPDH inhibitors.

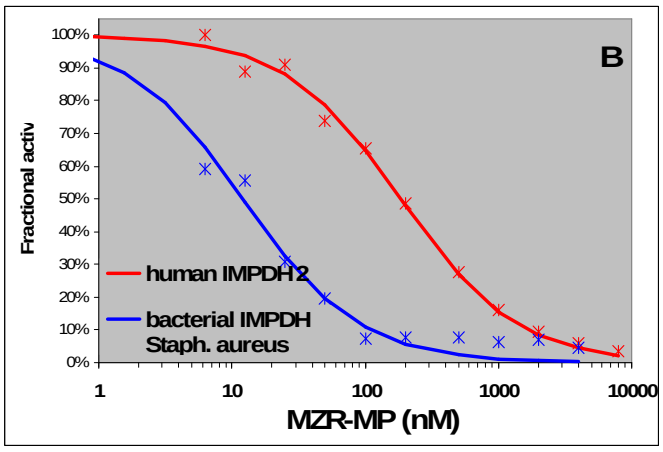
Unit Definition:
One unit of IMPDH converts 1.0 µmole of IMP and NAD to XMP and NADH per minute at pH 8 at 37°C.



Specific Activity:
≥ 0.3 unit/mg protein.

Purity: controlled by 12%AA SDS-PAGE.





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 KH_2PO_4 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:
[1]. L. Hedstrom and L. Gan (2006): IMP dehydrogenase: structural schizophrenia and an unusual base *Curr. Opin. Chem. Biol.* 10(5), 520-525.
[2]. Zhang R, Evans G, Rotella FJ, Westbrook EM, Beno D, Huberman E, Joachimiak A,Collart FR. Characteristics and crystal structure of bacterial inosine-5'-monophosphate dehydrogenase. *Biochemistry* (1999) 13;38(15):4691-700.

Related products:

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

Coupled nucleoside kinase - IMPDH II assay

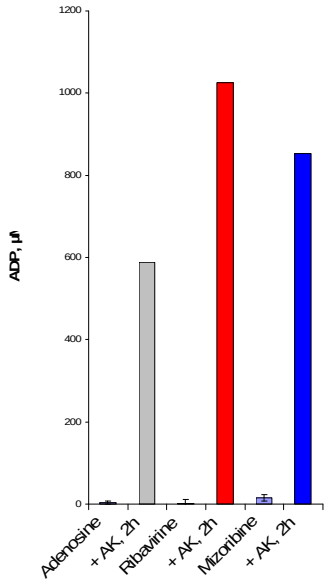
IMPORTANT: Client-specified alterations can be accommodated.

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.

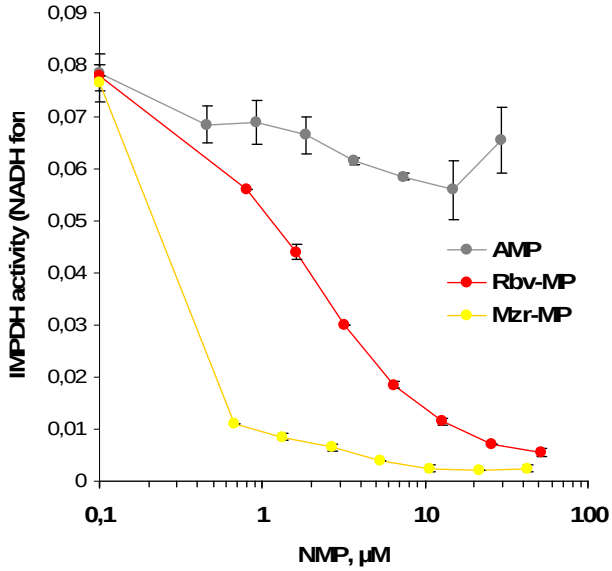
Aim: For rapid evaluation of monophosphate forms of nucleoside analogues as IMPDH inhibitors.

	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 **NOVOCIB**. Human recombinant IMPDH 2 was cloned from human cells, expressed in *E. coli* and purified by **NOVOCIB** (see sheet # E-Nov 1 for further information). The enzyme purity is controlled by SDS-PAGE, protein concentration is measured by Bradford method (Bio-Rad). A standard operating procedure (SOP) is followed to measure enzymatic activity.



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 antibovine 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.

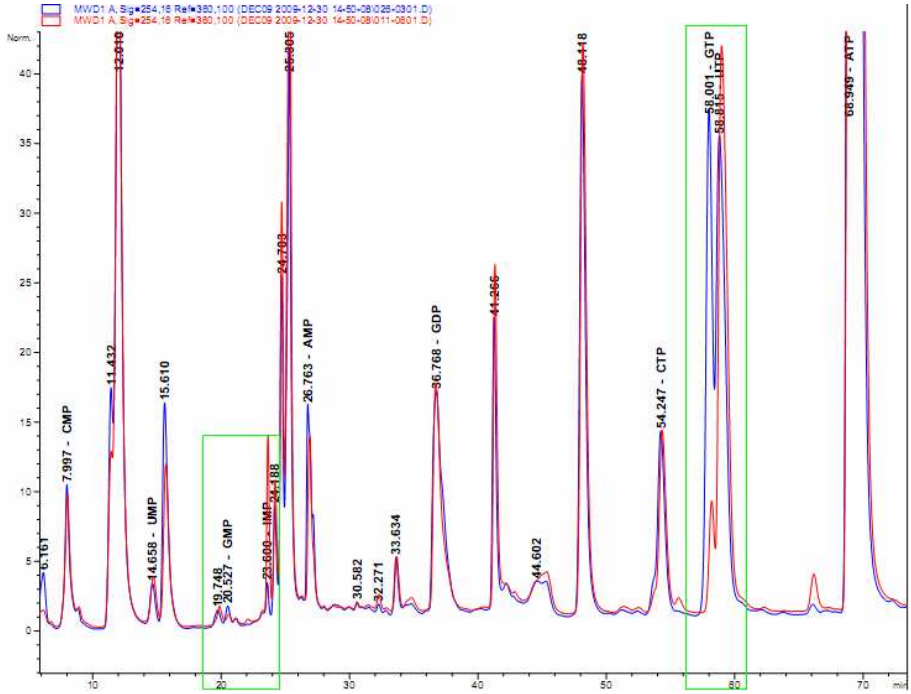
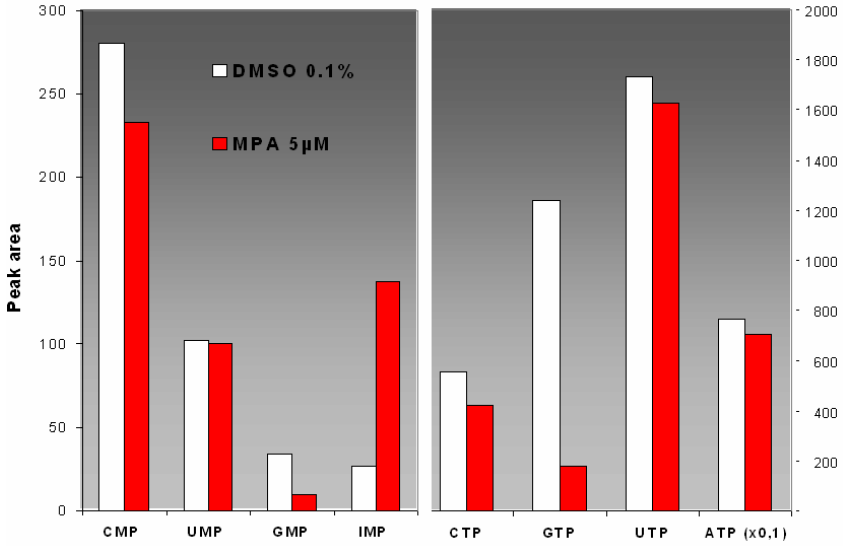


Figure 1. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.

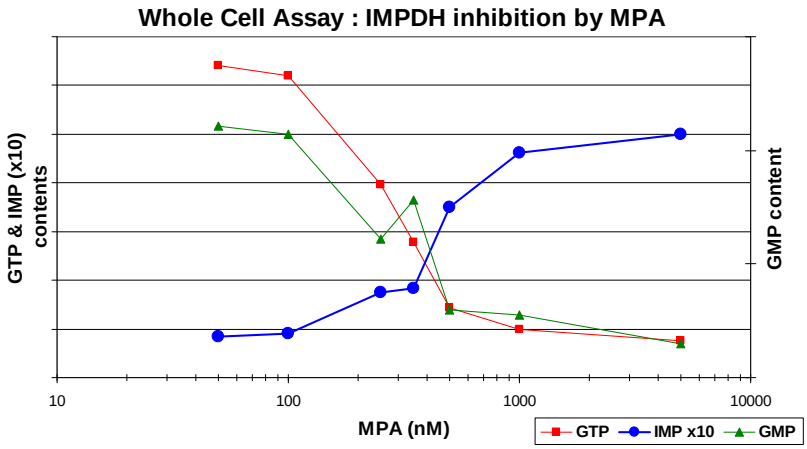


Concentration of nucleotides mono- and tri-phosphate in MPA- and DMSO-treated cells (measures as peak area, AU)

	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)

IC_{50} determination: Cellular GTP concentrations are plotted as a function of inhibitor concentration. IC_{50} is calculated using a standard four-parameter nonlinear regression analysis.
Plotting of minor nucleotides, such as IMP and GMP, is also available upon request.



2nd Example: Ribavirine (Rbv)

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

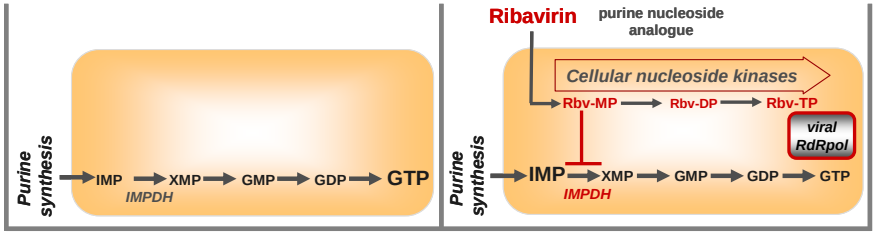


Figure 3. Modifications in cell-pool of nucleotides in Ribavirin-treated cells

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-pared 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.

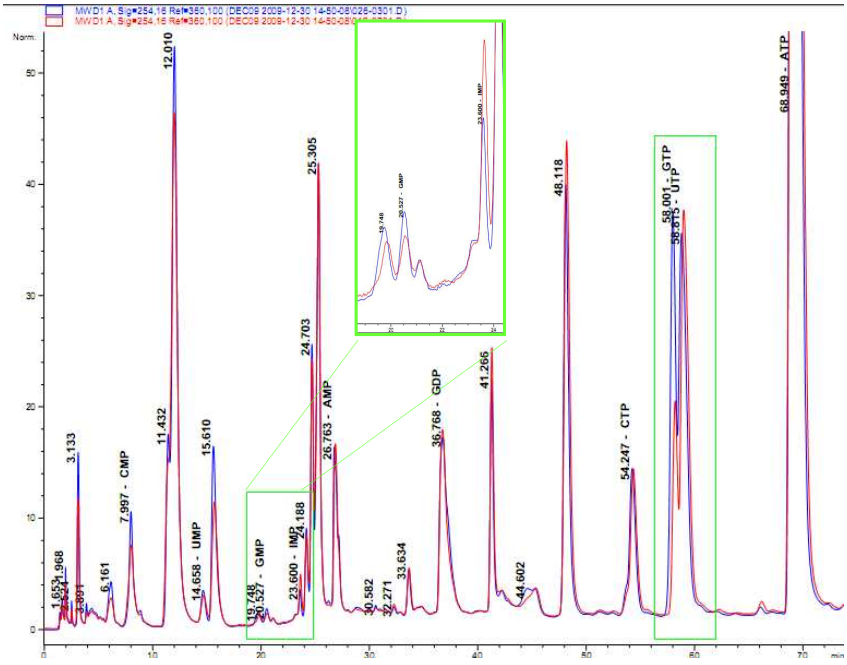
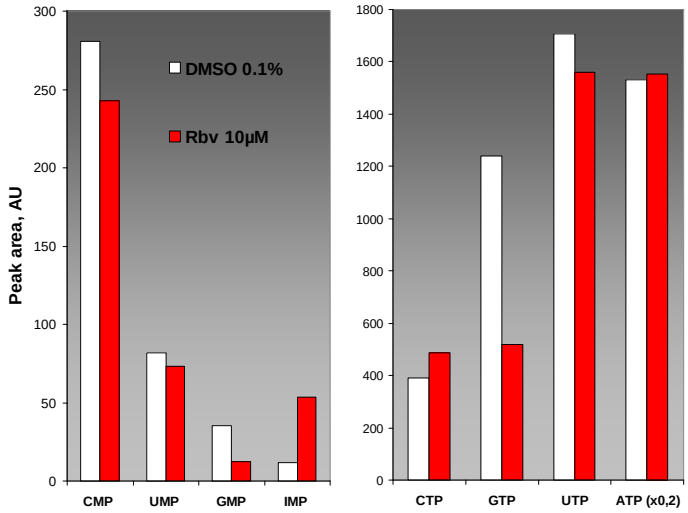


Figure 4. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 10µM Rbv (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.



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% CO_2 at 37°C in DMEM medium supplemented with 2mM L-glu tamine, 10% heat-inactivated fetal bovine serum and streptromycin-penicillin. Exponentially grown Huh-7 cells are seeded at ~$6x10^5$ cells per 10cm cell-culture dish. After 48h of growth, the culture medium is replaced with fresh FCS-supplemented medium followed by addition of 10µL of DMSO or DMSO-dissolved compound.

Extraction of nucleotides and deoxynucleotides - Sample preparation

The nucleotides are extracted from cell monolayers by addition of 3 ml per dish of ice-clod 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 wavelenght and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.

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

5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides[1] with slight modifications as follows.

HPLC calibration, peak identification and quantification

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

[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