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

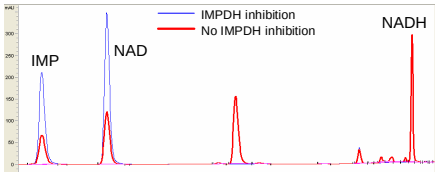
Controls are done in duplicate. If an additional microplate is needed, it includes the complete set of controls (in duplicate). Additional concentrations of inhibitor can be tested.

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)

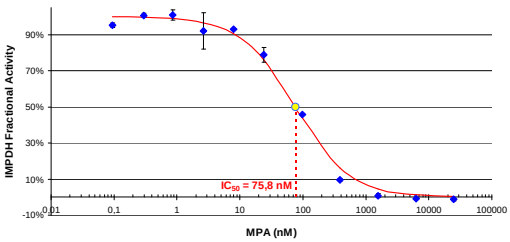
*Solubility in the reaction buffer must be checked before performing the assay.

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…).*