**Malachite Green Enzyme assay, NUDIX family, dNTP hydrolases, 384-well**

**Version: 1.0**

**Version Date: April 2021**

**1. Rationale/Aim**

*Purpose: Determine IC50, Kd and other biochemical parameters of Compounds or Substrates binding to Phosphate hydrolyzing enzymes listed below*

*Principle: Upon assay progression tri- and diphosphate substrate groups are hydrolyzed by the enzymes. This process releases inorganic phosphate groups which are forming a complex with the Malachite green molybdate reagent. The complex absorbs wavelength of 620-640 nm and can be used to indirectly quantify released phosphate and thus measure substrate turnover.*

*Benefits: The assay has high specificity and tends to pick up low numbers of false positives.*

**2. Experimental conditions**

***2.1 Key Requirement:***

*Plate Reader (reading 630 nm), MultiDrop (optional), Acoustic dispenser (optional), pipettes, Assay Plates: Nunc 262160 (clear, 384-well); Compound Plates: Greiner 781280 (PP, V-bottom; 384-wll); Echo Plates: Labcyte LP-0200; 384LDV; Assay buffer (see below individually); Protein (see below); Substrate (see below); Malachite Green Solution: Malachite Green Carbinol hydrochloride (Sigma #M9636) – 3.2 mM in in H2SO4 (60 ml conc. H2SO4 + 300 ml MilliQ H2O).*

***2.2 Key Resources Table:***

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| --- | --- | --- |
| *Reagents (items)* | *Suppliers* | *Cat. No.* |
| *Substrates:* |  |  |
| *dGTP* | *Jena BioScience* | *NU-424S, NU-424L* |
| *dCTP* | *Jena BioSicence* | *NU-1002L, NU-1002-10ML* |
| *AP4A* | *Jena Bioscience* | *NU-507S, NU-507L* |
| *ADPR* | *Toronto Research Chemicals* | *A207620* |
| *beta-NADH* | *Sigma* | *N-7004* |
| *UDP Galactose* | *BioSynth* | *MU58246* |
| *Salts and Additives:* |  |  |
| *Tris-Acetate* | *abcr GmbH* | *AB336704* |
| *Sodium Chloride* | *abcr GmbH* | *AB121999* |
| *Magnesium acetate* | *Biosynth* | *FM31347* |
| *Tween 20* | *abcr GmbH* | *AB252047* |
| *DTT* | *abcr GmbH* | *AB121727* |
| *Potassium chloride* | *abcr GmbH* | *AB119363* |
| *TCEP* | *abcr GmbH* | *AB121644* |
| *Enzymes:* |  |  |
| *E. coli pyrophosphatase (PPase)* | *Sigma* | *I5907* |
| *Alkaline pyrophosphatase from bovine intestinal mucosa*  *(BIP)* | *Sigma* | *P5521* |
| *NUDT1* | *Prospecbio* | *ENZ-010* |
| *NUDT2* | *Prospecbio* | *ENZ-063* |
| *NUDT5* | *Prospecbio* | *ENZ-547* |
| *NUDT9* | *Prospecbio* | *ENZ-137* |
| *NUDT12* | *fisher scientific* | *16185383* |
| *NUDT14* | *Prospecbio* | *ENZ-691* |
| *NUDT15* | *internal* |  |
| *NUDT18* | *internal* |  |
| *NUDT22* | *internal* |  |
| *dCTPase* | *Prospecbio* | *ENZ-234* |
| *dITPase* | *internal* |  |
| *dUTPase* | *Prospec* | *ENZ-568* |

**3. Protocol**

***Assay Preparation***

*Prepare a compound plate map for dose-responses according to the layout below. For this purpose, ideally, acoustic dispensing is used. The required compound source plate is prepared as follows:*

*Column 1,* ***10 mM****: 20 µl of 10 mM*

*Column 2****, 0.05 mM****: 1 µl of 1 mM + 19 µl DMSO*

*Column 3,* ***1 mM****: 2 µl of 10 mM + 18 µl DMSO*

*Column 4****, 0.005 mM****: 2 µl of 0.05 mM + 18 µl DMSO*

*Transfer these dilutions to Echo plates and dispense dose-response curves of compounds in assay plates with Echo dispenser. Seal resulting plates and keep in fridge until use.*

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|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| A | DR compound 1 | | | | | | | | | | | pos ctrl  ↓ | DR compound 1 | | | | | | | | | | | neg ctrl  ↓ |
| B | DR compound 2 | | | | | | | | | | | DR compound 2 | | | | | | | | | | |
| C | DR compound 3 | | | | | | | | | | | DR compound 4 | | | | | | | | | | |
| D | DR compound 4 | | | | | | | | | | | DR compound 4 | | | | | | | | | | |
| E | DR compound 5 | | | | | | | | | | | DR compound 5 | | | | | | | | | | |
| F | DR compound 6 | | | | | | | | | | | DR compound 6 | | | | | | | | | | |
| G | DR compound 7 | | | | | | | | | | | DR compound 7 | | | | | | | | | | |
| H | DR compound 8 | | | | | | | | | | | DR compound 8 | | | | | | | | | | |
| I | DR compound 9 | | | | | | | | | | | DR compound 9 | | | | | | | | | | |
| J | DR compound 10 | | | | | | | | | | | DR compound 10 | | | | | | | | | | |
| K | DR compound 11 | | | | | | | | | | | DR compound 11 | | | | | | | | | | |
| L | DR compound 12 | | | | | | | | | | | DR compound 12 | | | | | | | | | | |
| M | DR compound 13 | | | | | | | | | | | DR compound 13 | | | | | | | | | | |
| N | DR compound 14 | | | | | | | | | | | DR compound 14 | | | | | | | | | | |
| O | DR compound 15 | | | | | | | | | | | DR compound 15 | | | | | | | | | | |
| P | DR known inhibitor\* | | | | | | | | | | | DR known inhibitor\* | | | | | | | | | | |

*\* ideally, positive compound control - if not known, may be filled with DR compound 16*

***Assay***

*To allow transferable and comparable results, it is strongly recommended to use a Multidrop for dispensing of substrate and enzyme solution. Follow the tables below for the specific enzyme used before continuing with steps 1-5 further down. Prepare buffer, substrate and enzyme solution as mentioned below. Have Malachite Green Solution ready: Malachite Green Carbinol hydrochloride (Sigma #M9636) – 3.2 mM in in H2SO4 (60 ml conc. H2SO4 + 300 ml MilliQ H2O).*

|  |  |  |  |
| --- | --- | --- | --- |
| **NUDT1** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| Pyrophosphatase | 5000 U/ml | 0,4 U/ml | 0,2 U/ml |
| NUDT1 |  | 9.5 nM | 4.75 nM |
| dGTP | 100 mM | 200 µM | 100 µM |
| Reaction time: 15 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT2** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM TCEP | Stock concentration | Working solution | Final concentration |
| Phosphatase, bovine | 10000 U/mL | 20 U/mL | 10 U/mL |
| NUDT2 |  | 8 nM | 4 nM |
| AP4A | 60 mM | 8 µM | 4 µM |
| Reaction time: 15 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT5** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| BIP | 10000 U/mL | 20 U/mL | 10 U/mL |
| NUDT5 |  | 12 nM | 6 nM |
| ADPR | 100 mM | 100 µM | 50 µM |
| Reaction time: 15 min RT |  |  |  |

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| **NUDT9** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| BIP | 10000 U/mL | 20 U/mL | 10 U/mL |
| NUDT9 |  | 70 nM | 35 nM |
| ADPR | 100 mM | 100 µM | 50 µM |
| Reaction time: 15 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT12** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| BIP | 10000 U/mL | 20 U/mL | 10 U/mL |
| NUDT12 |  | 40 nM | 20 nM |
| AP4A | 100 mM | 100 µM | 50 µM |
| Reaction time: 30 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT14** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| BIP | 10000 U/mL | 20 U/mL | 10 U/mL |
| NUDT14 |  | 4 nM | 2 nM |
| ADPR | 100 mM | 100 µM | 50 µM |
| Reaction time: 15 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT15** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| Pyrophosphatase | 5000 U/ml | 0.4 U/ml | 0.2 U/ml |
| NUDT15 |  | 16 nM | 8 nM |
| dGTP | 100 mM | 200 µM | 100 µM |
| Reaction time: 15 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT18** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  1mM DTT  0.01% Tween-20 | Stock concentration | Working solution | Final concentration |
| PPase | 10000 U/mL | 0.8 U/mL | 0.2 U/mL |
| NUDT18 |  | 20 nM | 10 nM |
| dGTP | 10 mM | 200 µM | 100 µM |
| Reaction time: 10 min RT |  |  |  |

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| --- | --- | --- | --- |
| **NUDT22** 100 mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| BIP | 10000 U/ml | 20 U/ml | 10 U/ml |
| NUDT22 |  | 30 nM | 15 nM |
| UDP galactose |  | 100 µM | 50 µM |
| Reaction time: 30 min RT |  |  |  |

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| --- | --- | --- | --- |
| **dCTPase** 100mM Tris-acetate, pH 8.0  100mM KCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| Pyrophosphatase | 5000 U/ml | 0.4 U/ml | 0.2 U/ml |
| dCTPase |  | 70 nM | 35 nM |
| dCTP |  | 70 µM | 35 µM |
| Reaction time: 20 min RT |  |  |  |

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| --- | --- | --- | --- |
| **ITPase** 100 mM Tris-acetate, pH 8.0  50mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| Pyrophosphatase | 9170 U/ml | 0,4 U/ml | 0,2 U/ml |
| ITPase |  | 0,2 nM | 0,1 nM |
| ITP |  | 50 µM | 25 µM |
| Reaction time: 20 min RT |  |  |  |

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| **dUTPase** 100mM Tris-acetate, pH 8.0  40mM NaCl  10mM MgAc  0.005% Tween-20  1mM DTT | Stock concentration | Working solution | Final concentration |
| Pyrophosphatase | 9170 U/ml | 0,4 U/ml | 0,2 U/ml |
| UTPase |  | 2,1 nM | 1,05 nM |
| dUTP |  | 25 µM | 12,5 µM |
| Reaction time: 20 min RT |  |  |  |

1. Add “no enzyme control” (aka buffer only) in column 24, 25 µl/well with Multidrop.
2. Add enzyme working solution to all columns except 24: 25 µl/well with Multidrop. Preincubate 10 min RT. Spin in centrifuge at 1000g.
3. Add substrate working solution: 25 µl/well with Multidrop. Incubation 15-30 min at RT. Spin in centrifuge at 1000g.
4. Add Malachite Green reagent: 10 µl/well with Multidrop. Incubate 15 min or as indicated below at RT. Spin in centrifuge at 1000g.
5. Read absorbance at 630 nM, Malachite Green program.

## *Results*

Import the raw data into the template for Malachite Green assays (Malachite Green\_Template) following the instructions in the template (XLfit installation necessary for drawing of curves).

Check the quality of the assay considering z’ values and signal to background for each plate. Evaluate the curves according to “Pharmacology data reporting guidelines”. Check the values for the reference compound. If the quality of the assay is satisfying and the IC50 values for the reference compound are in the accepted range, report the IC50 values for the test compounds to the database.