**Protocol biochemical enzymatic assay for DNA glycosylases**

**Version: 1.0**

**Version Date: April 2021**

**1. Rationale/Aim**

*Purpose: Determine IC50, Kd and other biochemical parameters of Compounds or Substrates binding to DNA glycosylases*

*Principle: DNA glycosylases recognize a scope of nucleobase oxidation and alkylation in DNA and excise them. The rational of the assay is the incorporation of these substrates in a DNA Oligo with an Fluorophore attached downstream of the substrate. When the DNA substrate is not excised, i.e. the enzyme is inhibited, the DNA is intact and a Quencher placed in the complementary strand, opposite of the fluorophore, prevents excitation of the fluorophore. When no inhibitor is present, the substrate is removed from the DNA and a strand break is generated. This removes the quencher from the fluorophore and emission can be measured at 594 nM.*

*Benefits and Limitations: All assay turnovers can be assessed continuously or at single time points – useful for kinetics. High-throughput screen applicable. The assay is susceptible for auto-fluorescent and DNA intercalating compounds and a counter screen should be performed in three relevant concentrations. NEIL1 is used in a single turnover assay, as the enzyme is trapped on the excised abasic site. Assays for OGG1, UNG2, TDG, SMUG1 and MPG are APE1 coupled for (faster) turnover. A counter-screen should be performed for APE1. OGG1 has weak strand incision (AP-lyase) activity and can alternatively be run with longer incubation time.*

**2. Experimental conditions**

***2.1 Key Requirement:***

*Plate Reader (excitation 485 nM, emission 535 nm), Echo Dispenser (optional), MultiDrop (optional), pipettes, Assay buffer (see below individually); Protein (see below); Substrate (see below DNA); substrates are custom synthesis and in the range of 300€-1000€ (depends on synthesis scale), Assay Plates black 384-well plates (black OptiPlates, PerkinElmer); Compound Plates: Greiner 781280 (PP, V-bottom; 384-wll); Echo Plates: Labcyte LP-0200; 384LDV; Assay buffer (see below individually*

***2.2 Key Resources Table:***

|  |  |  |
| --- | --- | --- |
| *Reagents (items)* | *Suppliers* | *Cat. No.* |
| *Substrates:* |  |  |
| UNG2:5′-FAM-TCTG CCA UCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG ATG GCA GT-Dab-3 | *ATDBio, alternatively IDT* | *custom* |
| *NEIL1*5′-FAM-TCTG CCA YCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG CTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *OGG1*5′-FAM-TCTG CCA XCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG CTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *SMUG1*5′-FAM-TCTG CCA UCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG GTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *MPG*5′-FAM-TCTG CCA ICA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG TTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *TDG*5′-FAM-TCTG CCA UCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG GTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *APE1*5′-FAM-TCTG CCA ZCA CTG CGT CGA CCT G-3′5′-CAG GTC GAC GCA GTG GTG GCA GT-Dab-3′ | *ATDBio, alternatively IDT* | *custom* |
| *Salts and Additives* |  |  |
| *Tris-HCl* | *abcr GmbH* | *AB119983* |
| *Sodium Chloride* | *abcr GmbH* | *AB121999* |
| *Magnesium Chloride* | *abcr GmbH* | *AB202972* |
| *Potassium chloride* | *abcr GmbH* | *AB119363* |
| *EDTA Trisodium salt* | *abcr GmbH* | *AB436025* |
| *Tween 20* | *abcr GmbH* | *AB252047* |
| *DTT* | *abcr GmbH* | *AB121727* |
| *Gelatine* | *Sigma* | *G7765* |
| *Enzymes* |  |  |
| *NEIL1* | *internal* | *psfNEIL1-h002* |
| *OGG1* | *internal* |  |
| *UNG2* | *internal* |  |
| *APE1* | *internal* |  |
| *TDG* | *internal* |  |
| *MPG* | *New England Biolabs* | *M0313S* |
| *SMUG1* | *New England Biolabs* | *M0336S* |

*Fam – 6-Carboxyfluorescin, Dab – Dabcyl Quencher-3’, U – Uracil, Y – Thymine glycol, X- 8-oxoA, I – Inosine, Z – abascis site analogue (alternative names: THF; 1’,2’-Dideoxyribose; dSpacer).*

**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.*

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 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. Prepare buffer and enzyme solution as mentioned below.*

*Substrate Annealing:*

*To generate substrate stocks, dissolve the complementary DNA strands individually in 25mM Tris-HCl (pH 8.0), 50 mM NaCl and 2mM MgCl2 as stem buffer. Lift a ratio of 1:1.25 of Fam and Dabcyl modification containing stocks and combine in a fresh Tube to the corresponding stock concentration mentioned below. Place the tube in a preheated block at 95°C, remove the heated container and let the Tube slowly cool to room temperature. Store the resulting stock solutions in a cold (-20°C) dark place.*

*Bifunctional DNA glycosylases:*

*Bifunctional DNA glycosylases possess glycosylase and AP-lyase activity. Thus, they can perfom strand incision themselves and do not to be coupled with APE1.*

|  |  |  |  |
| --- | --- | --- | --- |
| **NEIL1**25mM Tris-HCl, pH 8.025mM KCl0.0025% Tween-201mM EDTA | Stock concentration | Working solution | Final concentration |
| NEIL1 | 167 µM | 200 nM | 40 nM |
| substrate | 10 µM | 25 nM | 20 nM |
| Reaction time: read at 0, 8, 15 and 30 min RT |  |  |  |

1. *Add “no enzyme control” (aka buffer only) in column 24, 10 µl/well with Multidrop.*
2. *Add enzyme working solution to all columns except 24: 10 µl/well with Multidrop. Preincubate 10 min RT. Spin in centrifuge at 1000g.*
3. *Add substrate working solution: 40 µl/well with Multidrop. Spin in centrifuge at 1000g.*
4. *Read excitation 485 nM, emission 535 nm at plate reader at time points 0, 8, 15 and 30 min. Proceed with Results below.*

*Monofunctional glycosylases:*

*Monofunctional DNA glycosylases possess only glycosylase activity. Thus, for a functioning strand incision assay, they are coupled with APE1. This in turn requires an APE1 assay for selectivity and counter screening (described further down). OGG1 has a residual AP-lyase activity and may perform β-elimination itself in vitro. However, here the assay turnover is very slow and APE1 is required for large throughput of substances.*

|  |  |  |  |
| --- | --- | --- | --- |
| **OGG1**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0022% Tween-20only protein stocks:2.5mM DTT50 µL fish gelatin per 10mL buffer | Stock concentration | Working solution | Final concentration |
| OGG1 | 4.05 µM | 202.5 pM | 40.5 pM |
| substrate | 10 µM | 12.5 nM | 10 nM |
| APE1 | 10 µM | 10 nM | 2nM |
| Reaction time: incubation overnight |  |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **UNG2**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0025% Tween-20 | Stock concentration | Working solution | Final concentration |
| UNG2 | 1 µM | 1.5 nM | 300 pM |
| substrate | 50 µM | 12.5 nM | 10 nM |
| APE1 | 10 µM | 10 nM | 2 nM |
| Reaction time: read at 0, 8, 15 and 30 min RT |  |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **TDG**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0025% Tween-20 | Stock concentration | Working solution | Final concentration |
| TDG | 1 µM | 125 nM | 25 nM |
| substrate | 50 µM | 12.5 nM | 10 nM |
| APE1 | 10 µM | 10 nM | 2 nM |
| Reaction time: read at 0, 8, 15 and 30 min RT |  |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **MPG**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0025% Tween-20 | Stock concentration | Working solution | Final concentration |
| MPG | 10000 U/mL | 1.5 U | 0.3 U |
| substrate | 50 µM | 12.5 nM | 10 nM |
| APE1 | 10 µM | 10 nM | 2 nM |
| Reaction time: read at 0, 8, 15 and 30 min RT |  |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **SMUG1**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0025% Tween-20 | Stock concentration | Working solution | Final concentration |
| SMUG1 | 5000 U/mL | 1.25 U/mL | 0.25 U/mL |
| substrate | 50 µM | 468.75 nM | 375 nM |
| APE1 | 10 µM | 10 nM | 2 nM |
| Reaction time: read at 0, 8, 15 and 30 min RT |  |  |  |

1. *Add “no enzyme control” (aka buffer only) in column 24, 10 µl/well with Multidrop.*
2. *Add enzyme working solution (containing DNA glycosylase and APE1) to all columns except 24: 10 µl/well with Multidrop. Preincubate 10 min RT. Spin in centrifuge at 1000g.*
3. *Add substrate working solution: 40 µl/well with Multidrop. Spin in centrifuge at 1000g.*
4. *Read excitation 485 nM, emission 535 nm at plate reader at time points 0, 8, 15 and 30 min (or overnight). Proceed with Results below.*

*Counter screen DNA glycosylase (APE1):*

*APE1 function is screened on an abasic site mimic, which is stable and selective for AP-lyase function.*

|  |  |  |  |
| --- | --- | --- | --- |
| **APE1**25mM Tris-HCl, pH 8.015mM NaCl2mM MgCl20.0022% Tween-20enzyme buffer only:2.5mM DTT50µL fish gelatin per 10 mL buffer | Stock concentration | Working solution | Final concentration |
| APE1 | 10 µM | 0.625 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 |  |  |  |

1. *Add “no enzyme control” (aka buffer only) in column 24, 10 µl/well with Multidrop.*
2. *Add enzyme working solution to all columns except 24: 10 µl/well with Multidrop. Preincubate 10 min RT. Spin in centrifuge at 1000g.*
3. *Add substrate working solution: 40 µl/well with Multidrop. Spin in centrifuge at 1000g.*
4. *Read excitation 485 nM, emission 535 nm at plate reader at time points 0, 8, 15 and 30 min. Proceed with Results below.*

## ***Results***

*Import the raw data into the template for DNA glycosylase assays (DNA\_Glycosylase\_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.*