Protocol of ESRE HeLa Cell-based Assay for High-throughput Screening

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| **DOCUMENT:** |  | ESRE\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of ESRE HeLa Cell-based Assay for High-throughput Screening |

**ASSAY RFERENCES:**

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| --- | --- | --- | --- | --- | --- | --- |
| Assay Target | Cell Lines | Species | Tissue of Origin | Assay Readout | Assay Provider | Toxicity Pathway |
| ATF6  (Recombinant) | HeLa | Human | Cervix | Beta lactamase reporter | Invitrogen | Stress response |

**QUALITY CONTROL PRECAUTIONS:**

1. -Cell culture is maintained by passaging twice a week and should not reach more than 90% confluence

2. -The assay should be performed in black-clear bottom 1536 well plates, so the bottom of the plates should not be touched

**MATERIALS and INSTRUMENTS:**

|  |  |  |
| --- | --- | --- |
| Supplies/Medium/Reagent | Manufacturer | Vender/Catalog Number |
| -DMEM+Glutamax | -Invitrogen | -Invitrogen/10569 |
| -Opti-MEM | -Invitrogen | -Invitrogen/11058 |
| -Dialyzed FBS | -Invitrogen | -Invitrogen/26400 |
| -NEAA | -Invitrogen | -Invitrogen/11140 |
| -Sodium pyruvate | -Invitrogen | -Invitrogen/11360 |
| -HEPES | -Invitrogen | -Invitrogen/15630 |
| -Penn-strep | -Invitrogen | -Invitrogen/15140 |
| -Blasticidin S HCl | -Invitrogen | -Invitrogen/A11139-03 |
| -Recovery Cell Culture Freezing Medium | -Invitrogen | -Invitrogen/12648 |
| -0.05% Trypsin-EDTA | -Invitrogen | -Invitrogen/25300 |
| -17-Allylamino-geldanamycin (17-AAG) | -LC Laboratories | -LC Laboratories/A-6880 |
| -Tetraoctyl ammonium bromide | -Sigma | -Sigma/294136 |
| -LiveBLAzer B/G FRET substrate | -Invitrogen | -Invitrogen/K1028 |
| -CellTiter-Glo One Solution Assay | -Promega | -Promega/G8462 |
| -Black-clear bottom 1536 well plates | -Greiner | -Greiner/789092F |
| -BioRAPTR FRD dispenser | -Beckman Coulter | -Beckman Coulter |
| -Multidrop COMBI | -Thermo Electron Corporation | -Thermo Electron Corporation |
| -Envision Plate Reader | -Perkin Elmer | -Perkin Elmer |
| -ViewLux Plate Reader | -Perkin Elmer | -Perkin Elmer |
| -17-AAG or 17-Allylamino-geldanamycin  (Agonist control compound) | -LC Laboratories | -LC Laboratories/A-6880 |

**PROCEDURE:**

1. Cell handling:

1.1. Media Required:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Component | Growth Medium | Assay Medium | Thaw Medium | Freezing Medium |
| -DMEM+Glutamax | -90% | - | -90% | - |
| -Opti-MEM | - | -99.5% | - | - |
| -Dialyzed FBS | -10% | -0.5% | -10% | - |
| -NEAA | -0.1mM | -0.1mM | -0.1mM | - |
| -Sodium pyruvate | - | -1mM | - | - |
| -HEPES | -25mM | - | -25mM | - |
| -Penn-strep | -100U/ml-100ug/ml | -100U/ml-100ug/ml | -100U/ml-100ug/ml | - |
| -Blasticidin S HCl | -5 ug/mL | - | - | - |
| -Recovery Cell Culture Freezing Medium | - | - | - | -100% |

1.2. Thawing method

1.2.1 -1ml frozen cells of ESRE bla HeLa were taken in pre-warmed 10ml of thaw medium for centrifuging.

1.2.2 -2-3ml of the thaw medium is taken to resuspend the pellet

1.2.3 -The cells were seeded in T-75 flask at 2 million cells

1.3. Propagation method

1.3.1 -Rinse the cells with DPBS and detach them by using 0.05% Trypsin and centrifuge

1.3.2 -The cells are further passaged at a density of 3 million cells per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in assay medium at a density of 0.25 X 10^6 cells/mL

2.2 -Plate the cells in black-clear bottom 1536 well plate at 1500/well/6uL of assay medium through 8 tip of a plate dispenser (Multi drop)

2.3 -Incubate at 37C for an overnight (18hrs)

2.4 -Transfer 23nL of compounds from the library collection and positive control through Pintool

2.5 -Incubate at 37C for 5hrs

2.6 -Add 1uL of CCF4 dye using a single tip of a plate dispenser (Bioraptr)

2.7 -Incubate at room temperature for 2hrs

2.8 -Read the fluorescence intensity through Envision plate reader

2.9 -Add 4uL of CellTiter-Glo reagent using a single tip of a plate dispenser (Bioraptr)

2.10 -Incubate at room temperature for 30 min

2.11 -Read the luminescence through ViewLux plate reader

3. Assay Performance

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| **EndoR**  **(17-AAG; Agonist control)** | **Online Validation**  **Agonist**  **(Mean ± SD)** | **Online Validation**  **Viability**  **(Mean ± SD)** |
| EC50 | 0.69 ± 0.05 μM  (n = 27) | NA |
| S/B | 2.95 ± 0.08 | 30.94 ± 2.07 |
| CV (%) ⃰ | 3.31 ± 0.23  (n = 18) | 8.57 ± 1.50  (n = 18) |
| Z’ | 0.70 ± 0.05 | 0.69 ± 0.11 |

⃰ CV values shown represent average of DMSO plates and low concentration plates only.