Protocol of AR-BLA HEK293 Cell-based Assay for High-throughput Screening

|  |  |  |
| --- | --- | --- |
| **DOCUMENT:** |  | AR-BLA\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of AR-BLA HEK293 Cell-based Assay for High-throughput Screening |

**ASSAY RFERENCES:**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Assay Target | Cell Lines | Species | Tissue of Origin | Assay Readout | Assay Provider | Toxicity Pathway |
| Androgen receptor : LBD  (Recombinant) | HEK293 | Rat (Androgen Receptor) | Embryonic kidney | Beta lactamase reporter | Invitrogen | NR signaling |

**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, high glucose | -Invitrogen | -Invitrogen/11965 |
| -Opti-MEM | -Invitrogen | -Invitrogen/11058 |
| -Dialyzed FBS | -Invitrogen | -Invitrogen/26400 |
| -HEPES | -Invitrogen | -Invitrogen/15630 |
| -NEAA | -Invitrogen | -Invitrogen/11140 |
| -Sodium pyruvate | -Invitrogen | -Invitrogen/11360 |
| -Penicillin and Streptomycin | -Invitrogen | -Invitrogen/15140 |
| -Hygromycin | -Invitrogen | -Invitrogen/10687 |
| -Zeocin | -Invitrogen | -Invitrogen/R250-01 |
| -0.05% Trypsin-EDTA | -Invitrogen | -Invitrogen/25300 |
| -Recovery Cell Culture Freezing Medium | -Invitrogen | -Invitrogen/12648 |
| -Black-clear bottom 1536 well plates | -Greiner | -Greiner/789092F |
| -LiveBLAzer B/G FRET substrate | -Invitrogen | -Invitrogen/K1028 |
| -CellTiter-Glo Assay Custom Solution | -Promega | -Promega/X2371 |
| -Multidrop COMBI | -Thermo Electron Corporation | -Thermo Electron Corporation |
| -BioRAPTR FRD dispenser | -Beckman Coulter | -Beckman Coulter |
| -Envision Plate Reader | -Perkin Elmer | -Perkin Elmer |
| -ViewLux Plate Reader | -Perkin Elmer | -Perkin Elmer |
| Cyproterone acetate  (Antagonist control compound) | -Sigma Aldrich | -Sigma Aldrich/C3412 |

**PROCEDURE:**

1. Cell handling:

1.1. Media Required:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Component | Growth Medium | Assay Medium | Thaw Medium | Freezing Medium |
| -DMEM, high glucose | -90% | - | -90% | - |
| -Opti-MEM | - | -90% | - | - |
| -Dialyzed FBS | -10% | -10% | -10% | - |
| -HEPES | -25mM | - | -25mM | - |
| -NEAA | -0.1mM | -0.1mM | -0.1mM | - |
| -Sodium pyruvate | -1mM | -1mM | -1mM | - |
| -Penicillin and Streptomycin | -100U/ml and 100ug/ml | -100U/ml and 100ug/ml | -100U/ml and 100ug/ml | - |
| -Hygromycin | -80ug/ml | - | - | - |
| -Zeocin | -80ug/ml | - | - | - |
| -Recovery Cell Culture Freezing Medium | - | - | - | -100% |

1.2. Thawing method

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

1.2.2 -Thaw medium is used to resuspend the pellet

1.2.3 -Seed the cells at 2 million per T-75 flask with thaw medium

1.3. Propagation method

1.3.1 -Detach the cells from the flask using 0.05% Trypsin

1.3.2 -The cells are re-seeded in T-225 flask at 3-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium

2.3 -Plate the cells in black-clear bottom 1536 well plate at 2000/well/6uL through 8 tip Multidrop plate dispenser

2.4 -Incubate at 37C for 5hrs

2.5 -Add 1uL of assay buffer by using single tip of a plate dispenser (Bioraptr) into bottom 1/3rd part of 2 and 3 columns

2.6 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.7 -Add 1uL of 10nM (final) R1881 by using single tip of a plate dispenser (Bioraptr) into all the wells except the buffer dispensed wells of bottom 1/3rd part of 2 and 3 columns

2.8 -Incubate at 37C for 16hrs

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

2.10 -Incubate at room temperature for 2hrs

2.11 -Read the fluorescence intensity through Envision plate reader

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

2.13 -Incubate at room temperature for 30min

2.14 -Read the luminescence through ViewLux plate reader

3. Assay Performance

|  |  |  |
| --- | --- | --- |
| **AR-bla**  **(Cyproterone acetate;**  **Antagonist control)** | **Online Validation**  **Antagonist**  **(Mean ± SD)** | **Online Validation**  **Viability**  **(Mean ± SD)** |
| IC50 | 1.85 ± 2.05 μM  (n = 27) | NA |
| S/B | 1.94 ± 0.06 | 131.80 ± 5.26 |
| CV (%) ⃰ | 6.67 ± 0.53  (n = 18) | 6.77 ± 0.46  (n = 18) |
| Z’ | 0.43 ± 0.08 | 0.84 ± 0.03 |

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