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

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| **DOCUMENT:** |  | ER-alpha-BLA\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of ER-alpha-BLA HEK293 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 |
| Estrogen receptor alpha: LBD  (Recombinant) | HEK293 | Human | 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. Culture medium should be replaced with Assay medium overnight prior to the assay

3. 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 |
| Phenol red-free DMEM | Invitrogen | Invitrogen/21063 |
| DMEM | Invitrogen | Invitrogen/11965 |
| Dialyzed FBS | Invitrogen | Invitrogen/26400 |
| Charcoal stripped FBS | Invitrogen | Invitrogen/12676 |
| NEAA | Invitrogen | Invitrogen/11140 |
| Sodium pyruvate | Invitrogen | Invitrogen/11360 |
| Penn-strep | Invitrogen | Invitrogen/15140 |
| Hygromycin B | Invitrogen | Invitrogen/10687 |
| Zeocin | Invitrogen | Invitrogen/R25001 |
| Recovery Cell Culture Freezing Medium | Invitrogen | Invitrogen/12648 |
| 0.05% Trypsin-EDTA | Invitrogen | Invitrogen/25300 |
| LiveBLAzer B/G FRET substrate | Invitrogen | Invitrogen/K1028 |
| CellTiter-Glo Assay Custom Solution | Promega | Promega / X2371 |
| 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 |
| 4-Hydroxytamoxifen  (Antagonist control compound) | Sigma | Sigma/H7904 |

**PROCEDURE:**

1. Cell handling:

1.1. Media Required:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Component | Growth Medium | Assay Medium | Thaw Medium | Freezing Medium |
| Phenol red-free DMEM | - | 98% | - | - |
| DMEM | 90% | - | 90% | - |
| Dialyzed FBS | 10% | - | 10% | - |
| Charcoal stripped FBS | - | 2% | - | - |
| NEAA | 0.1mM | 0.1mM | 0.1mM | - |
| Sodium pyruvate | 1mM | 1mM | 1mM | - |
| Penn-strep | 100U/ml-100ug/ml | 100U/ml-100ug/ml | 100U/ml-100ug/ml | - |
| Hygromycin B | 80ug/ml | - | - | - |
| Zeocin | 100ug/ml | - | - | - |
| Recovery Cell Culture Freezing Medium | - | - | - | 100% |

1.2. Thawing method

1.2.1 1ml frozen cells of ERalpha-bla 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 The cells are detached using 0.05% Trypsin

1.3.2 Cells are further passaged at a density of 4-5 million cells per T-225 flask

2. Assay Protocol

2.1 Rinse the cells twice with DPBS and detach them using 0.05% Trypsin and centrifuge

2.2 Resuspend the pellet with assay buffer

2.3 Plate the cells in black-clear bottom 1536 well plate at 5000/well/5uL through 8 tip plate dispenser (Multi drop)

2.4 Incubate at 37C for 5hrs

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

2.6 Add 1uL of assay buffer with or without 0.5nM (final) Beta-estradiol

2.7 Incubate at 37C for 18hrs

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

2.9 Incubate at room temperature for 2hrs

2.10 Read the fluorescence intensity through Envision plate reader

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

2.12 Incubate at room temperature for 30min

2.13 Read the luminescence through ViewLux plate reader

3. Assay Performance

|  |  |
| --- | --- |
| **ERα-bla**  **(4-Hydroxy Tamoxifen;**  **Antagonist control)** | **Online Validation**  **Antagonist**  **(Mean ± SD)** |
| IC50 | 0.01 ± 0.002 μM  (n = 27) |
| S/B | 2.31 ± 0.08 |
| CV (%) ⃰ | 4.71 ± 1.05  (n = 18) |
| Z’ | 0.68 ± 0.09 |

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