Protocol of VDR-BLA HEK 293T Cell-based Assay for High-throughput Screening

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| **DOCUMENT:** |  | VDR-BLA\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of VDR-BLA HEK 293T 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 |
| Vitamin D receptor: LBD  (Recombinant) | HEK 293T | 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. -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:**

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| --- | --- | --- |
| Supplies/Medium/Reagent | Manufacturer | Vender/Catalog Number |
| -Phenol red-free DMEM | -Invitrogen | -Invitrogen/21063 |
| -DMEM | -Invitrogen | -Invitrogen/10569 |
| -Dialyzed FBS | -Invitrogen | -Invitrogen/26400 |
| -Charcoal stripped FBS | -Invitrogen | -Invitrogen/12676 |
| -NEAA | -Invitrogen | -Invitrogen/11140 |
| -Sodium pyruvate | -Invitrogen | -Invitrogen/11360 |
| -HEPES | -Invitrogen | -Invitrogen/15630 |
| -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 |
| -Black-clear bottom 1536 well plates | -Greiner | -Greiner/789092F |
| -Vitamin D3, 1α, 25-Dihydroxy- (Calcitriol)  (Agonist control compound) | -EMD Millipore (Calbiochem) | -EMD Millipore (Calbiochem)/679101 |
| -Multidrop COMBI | -Thermo Electron Corporation | -Thermo Electron Corporation |
| -BioRAPTR FRD dispenser | -Beckman Coulter | -Beckman Coulter |
| -LiveBLAzer B/G FRET substrate | -Invitrogen | -Invitrogen/K1028 |
| -CellTiter-Glo(R) One Solution Assay | -Promega | -Promega/G8462 |
| -Envision Plate Reader | -Perkin Elmer | -Perkin Elmer |
| -ViewLux Plate Reader | -Perkin Elmer | -Perkin Elmer |

**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 | - | - |
| -HEPES | -25mM | - | -25mM | - |
| -Penn-strep | -100U/ml-100ug/ml | -100U/ml-100ug/ml | -100U/ml-100ug/ml | - |
| -Hygromycin B | -80ug/ml | - | - | - |
| -Zeocin | -80ug/ml | - | - | - |
| -Recovery Cell Culture Freezing Medium | - | - | - | -100% |

1.2. Thawing method

1.2.1 -1ml frozen cells of VDR-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 millions

1.3. Propagation method

1.3.1 -The cells are detached using 0.05% Trypsin

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

2. Assay Protocol

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

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 tips of a 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 3nM (final) 1α, 25-Dihydroxy-Vitamin D3 or assay medium on the top using two different tips of a plate dispenser (Bioraptr)

2.7 -Incubate at 37C for 16hrs

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

2.12 -Incubate at room temperature for 30 min

2.13 -Read the luminescence intensity through ViewLux plate reader

3. Assay Performance

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| **VDR-bla**  **(Antagonist control not available)** | **Online Validation**  **Antagonist**  **(Mean ± SD)** | **Online Validation**  **Viability**  **(Mean ± SD)** |
| IC50 | NA | NA |
| S/B | 1.75 ± 0.05 | 175.09 ± 41.02 |
| CV (%) ⃰ | 7.87 ± 0.43  (n = 18) | 14.86 ± 1.62  (n = 18) |
| Z’ | 0.46 ± 0.11 | 0.48 ± 0.08 |

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