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

DOCUMENT: VDR-BLA_TOX21_SLP_Version1.0

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ASSAY RFERENCES:

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:

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

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.