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

DOCUMENT: AR-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
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