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

DOCUMENT: ER-alpha-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
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.