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

DOCUMENT: SBE-BLA_TOX21_SLP_Version1.0

TITLE: Protocol of SBE-BLA HEK 293T Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Smad 2/3	HEK 293T	Human	Embryonic kidney cells	Beta-lactamase reporter	Invitrogen	TGF-beta/Smad signaling

QUALITY CONTROL PRECAUTIONS:

1. -Cell culture is maintained by passing cells twice a week and should not reach more than 90% confluence
2. -The assay should be performed in black-clear bottom 1536 well plates and the bottom of the plates should not be touched
3. –It is recommended to pass the cells for three passages after thawing before using them in the beta-lactamase assay.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
-DMEM	-Invitrogen	-Invitrogen/11965
-Dialyzed FBS	-Invitrogen	-Invitrogen/26400
-NEAA	-Invitrogen	-Invitrogen/11140
-Sodium pyruvate	-Invitrogen	-Invitrogen/11360
-HEPES	-Invitrogen	-Invitrogen/15630
-Penn-strep	-Invitrogen	-Invitrogen/15140
-Blastcidin	-Invitrogen	-Invitrogen/A11139-03
-Recovery Cell Culture Freezing Medium	-Invitrogen	-Invitrogen/12648
-0.05% Trypsin-EDTA	-Invitrogen	-Invitrogen/25300
-Black-clear bottom 1536 well plates	-Greiner	-Greiner/789092F

-Recombinant Human TGF-beta 1 Protein	-R & D Systems	-R & D Systems/240-B-002
- Tetraoctylammonium bromide	-Sigma-Aldrich	-Sigma-Aldrich/294136
-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
-DMEM	-90%	-90%	-90%	-
-Dialyzed FBS	-10%	-10%	-10%	-
-NEAA	-0.1mM	-0.1mM	-0.1mM	-
-Sodium pyruvate	-1mM	-1mM	-1mM	-
-HEPES	-25mM	-25mM	-25mM	-
-Penn-strep	-100U/ml-100ug/ml	-100U/ml-100ug/ml	-100U/ml-100ug/ml	-
-Blasticidin	-5ug/ml	-	-	-
-Recovery Cell Culture Freezing Medium	-	-	-	-100%

1.2. Thawing method

1.2.1 -1ml frozen cells of SBE-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 at a density of 2 million cells per T-75 flask in thaw medium

1.3. Propagation method

1.3.1 -The cells are detached using 0.05% Trypsin
1.3.2 -The cells are further passed at a density of 4-5 million cells per T-225 flask in culture medium

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 4000/well/6uL using 8-tip plate dispenser (Multi drop)
2.4 -Incubate at 37C for an overnight (18-20hr)
2.5 -Transfer 23nL of the compounds from the library collection and positive control using Pintool station
2.6 -Incubate at 37C for 5hr
2.7 -Add 1uL of CCF4 dye using a single tip of a plate dispenser (BioRAPTR)
2.8 -Incubate at room temperature for 2hr
2.9 -Read the fluorescence intensity through Envision plate reader (Excitation 405nm and Emissions at 460 and 530nms)
2.10 -Then add 4uL of CellTiter-Glo reagent using a single tip of a plate dispenser (BioRAPTR)
2.11 - Incubate at room temperature for 30min
2.12 -Read the luminescence intensity through ViewLux plate reader

3. Assay Performance

Online Validation	**SBE-bla HEK 293T Agonist (Mean ± SD)**	**SBE-bla HEK 293T Viability (Mean ± SD)**
AC_{50} (ng/mL)	0.19 ± 0.02 (n = 27)	N/A
S/B	2.37 ± 0.07	147.12 ± 8.42
CV (%)	4.91 ± 0.34* (n = 24)	7.88 ± 0.40* (n = 24)
Z’	0.68 ± 0.05	0.74 ± 0.09

*CV values shown represent average of all plates excluding high compound concentration plates.