

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

DOCUMENT: SBE-BLA_TOX21_SLP_Version1.0
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ASSAY REFERENCES:

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 -After compound addition, 1uL of 0.25ng/mL (final concentration) of recombinant human TGF-beta 1 and assay buffer was added using two separate tips of a plate dispenser (BioRAPTR)
- 2.7 -Incubate at 37C for 5hr
- 2.8 -Add 1uL of CCF4 dye using a single tip of a plate dispenser (BioRAPTR)
- 2.9 -Incubate at room temperature for 2hr
- 2.10 -Read the fluorescence intensity through Envision plate reader (Excitation 405nm and Emissions at 460 and 530nms)
- 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 30min
- 2.13 -Read the luminescence intensity through ViewLux plate reader

3. Assay Performance

Online Validation	SBE-bla HEK 293T Antagonist (Mean ± SD)	SBE-bla HEK 293T Viability (Mean ± SD)
AC50	N/A	N/A
S/B	2.19 ± 0.10	136.79 ± 10.40
CV (%)	6.92 ± 0.56* (n = 24)	6.67 ± 0.32* (n = 24)
Z'	0.56 ± 0.06	0.76 ± 0.04

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