Protocol of CRE-bla HEK 293T Cell-based Assay for High-throughput Screening (Antagonist mode)

DOCUMENT: CRE BLA_TOX21_SLP_Version1.0

TITLE: Protocol of CRE-bla HEK 293 Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
CRE LBD (Recombinant)	HEK 293	Human	Embryonic kidney cells	Beta-lactamase reporter	Invitrogen	NR signaling

QUALITY CONTROL PRECAUTIONS:

1. The cells should not be grown more than 80-85% confluence
2. The cell performance is affected if they are too confluent
3. Do not leave cells in Trypsin for more than 5 min at RT
4. Handle 1536 well plate black clear bottom plates carefully by sides

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM	Invitrogen	11965
Dialyzed FBS	Invitrogen	26400
Sodium Pyruvate	Invitrogen	11360
Pen-Strep	Invitrogen	15140
NEAA	Invitrogen	11140
HEPES	Invitrogen	15630
Blasticidin	Invitrogen	R21001
0.05% Trypsin-EDTA	Invitrogen	25300-054
Captan	Sigma	32054
Multidrop	Thermofisher	-
BiorapTR	Beckman Coulter	-
Envision Plate Reader	Perkin Elmer	-

LiveBLAzer B/G FRET substrate	Invitrogen	K1030

PROCEDURE:

1. Cell handling:
1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM	90%	90%	90%	-
Dialyzed FBS	10%	10%	10%	-
Sodium Pyruvate	1mM	1mM	-	-
Penn-strep	1%	1%	1%	-
HEPES	25 mM	25 mM	25 mM	-
NEAA	0.1 mM	0.1 mM	0.1 mM	-
Blasticidin	5 ug/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 ml of pre-warmed thaw medium into a 15 ml conical tube
1.2.2 -Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO
1.2.4 -Discard the supernatant and transfer the precipitated cells to T225 flask using 45 ml thawing medium or five-layer flask using 200ml thawing medium

1.3. Propagation method

1.3.1 -Detach the cells from the flask using 0.05% Trypsin-EDTA
1.3.2 -The cells are re-seeded in T-225 flask at 3 million or in five-layer flask at 15 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsin
2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust to the required cell density
2.3 -Plate the cells in black-clear bottom 1536 well plate at 2000/well/4µl for antagonist mode through 8 tip Multidrop plate dispenser
2.4 -Incubate for 18hrs at 37ºC / 95% Humidity / 5% CO2
2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool
2.6 -Add 1µl of buffer or 1µl of 0.25µM NKH477 to respective columns as per plate map for antagonist mode

2.7 -Incubate for 3hrs at 37ºC / 95% Humidity / 5% CO2
2.8 -Add 1µl of CCF4 (FRET Substarte) dye using a single tip plate dispenser (Bioraptr)
2.9 -Incubate at room temperature for 2hrs in dark
2.10 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamaze protocol optimized for this cell type
2.11 -Add 4µl of CellTiter Glo and Incubate at room temperature for 0.5hrs in dark
2.12 -Read on ViewLux protocol optimized for this cell type

3. Assay Performance

3.1 Performance of online validation:

CRE-bla HEK293 Antagonist (Captan)	Online Validation (Mean ± SD)	Online Validation Viability (Mean ± SD)
AC50	13.99 ± 1.25 µM (n =27)	47.76 ± 18.71
S/B	2.98 ± 0.11	139.20 ± 6.00
CV (%)	7.91 ± 1.03 (n = 24)	4.71 ± 0.24 (n = 24)
Z'	0.51 ± 0.07	0.86 ± 0.03

* CV values shown represent average of all assay plates excluding the top concentration plates.

3.2 Performance of online screening:

Assay	Active match	Inactive match	Inconclusive	Mismatch	AC50 fold change	Score
tox21-cre-antagonist-p1 ratio	5.33%	86.74%	7.80%	0.13%	1.33	89.32
tox21-cre-antagonist-p1 viability	3.39%	93.21%	3.38%	0.01%	1.35	96.60