

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

DOCUMENT: CRE BLA_TOX21_SLP_Version1.0

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

ASSAY REFERENCES:

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
Forskolin (NKH477)	Sigma	344281
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/5µl for agonist mode through 8 tip Multidrop plate dispenser

2.4 -Incubate for 18hrs at 37°C / 95% Humidity / 5% CO₂

2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintoal

2.6 -Incubate for 3hrs at 37°C / 95% Humidity / 5% CO₂

2.7 -Add 1µl of CCF4 (FRET Substarte) dye using a single tip plate dispenser (Bioraptr)

- 2.8 -Incubate at room temperature for 2hrs in dark
 2.9 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamase protocol optimized for this cell type
 2.10 -Add 4µl of CellTiter Glo and Incubate at room temperature for 0.5hrs in dark
 2.11 -Read on ViewLux protocol optimized for this cell type

3. Assay Performance

3.1 Performance of online validation:

CRE-bla HEK293 Agonist (NKH477)	Online Validation (Mean ± SD)	Online Validation Viability (Mean ± SD)
EC50	0.27 ± 0.06 µM (n =27)	N/A
S/B	4.16 ± 0.24	126.63 ± 6.13
CV (%)	6.59 ± 0.97 (n = 24)	4.71 ± 1.01 (n = 24)
Z'	0.82 ± 0.02	0.66 ± 0.04

* 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-agonist-p1_ratio	1.01%	98.20%	0.79%	0.00%	1.32	99.44
tox21-cre-agonist-p1_viability	2.66%	93.35%	3.97%	0.02%	1.41	94.65