Protocol of ARE-BLA HepG2 Cell-based Assay for High-throughput Screening

DOCUMENT: ARE-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
Nrf2/ARE (Recombinant)	HepG2	Human	Hepatocellular carcinoma	Beta-lactamase	Invitrogen	Stress response

QUALITY CONTROL PRECAUTIONS:

1. Cells should be grown to reach 60 to 75% confluence
2. Handle the 1536-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM with GlutaMAX	Invitrogen	10569
Fetal bovine serum, dialyzed	Invitrogen	26400
Nonessential amino acids (NEAA)	Invitrogen	11140
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140
DPBS	Invitrogen	14190
HEPES (1 M, pH 7.3)	Invitrogen	15630
0.25% Trypsin/EDTA	Invitrogen	25300
Blasticidin (antibiotic)	Invitrogen	R210
LiveBLAzer B/G FRET Loading Kit (Solution A, B and C)	Invitrogen	K1030
Solution D	Invitrogen	K1157
β-Naphthoflavone	Sigma	70415
Blackwall, clear-bottom, 1536-well assay plates	Greiner BioOne	789092-F

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
DMEM with GlutaMAX	90%	99%	90%	-
Dialyzed FBS	10%	1%	10%	-
NEAA	0.1 mM	0.1 mM	0.1 mM	-
HEPES (pH 7.3)	25 mM	25 mM	25 mM	-
Penicillin/Streptomycin	100U/mL/100µg/mL	100U/mL/100µg/mL	100U/mL/100µg/mL	-
Blasticidin (antibiotic)	5 ug/mL	-	-	-
Recovery Cell freezing medium	-	-	-	100%

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a T75 flask
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 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.
1.2.4 Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tub.
1.2.5 Centrifuge cells at 900 rpm for 4 minutes and resuspend in thaw medium.
1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator.
1.2.7 Switch to growth medium at first passage.

1.3. Propagation method

1.3.1 Aspirate medium, rinse once in DPBS, add 0.25% Trypsin/EDTA and swirl to coat the cell evenly.
1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.
1.3.3 Centrifuge cells at 900 rpm for 4 minutes and resuspend in growth medium.
1.3.4 Cell should be passage at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture in growth medium and resuspend in assay medium.
2.2 Dispense 2000 cells/5μL/well into 1536-well tissue treated black/clear bottom plates using a BioRAPTR dispenser.
2.3 After the cells were incubated at 37°C for 5 hours, 23 nL of positive controls or compounds were transferred to the assay plate by a PinTool resulting in a 217-fold dilution. The final compound concentration in the 5 μl assay volume ranged from 1.2 nM to 92 μM in 15 concentrations.
2.4 Incubate the plates for 16 hours at 37°C.
2.5 Add 1 μL of 6X LiveBLAzer-FRET B/G (CCF4-AM) Substrate Mixture to each well using a BioRAPTR dispenser and incubate the plate at room temperature for 2 hours.
2.6 Measure fluorescence intensity at 460 and 530 nm emission and 405 nm excitation by an Envision detector. Data is expressed as the ratio of 460nm/530nm emissions.

3. Assay Performance

ARE-bla Agonist (β-Naphthoflavone)	Online Validation (Mean ± SD)
EC50	1.47 ± 0.19 μM (n = 27)
S/B	3.08 ± 0.26 (n = 27)
CV (%)	10.63 ± 2.12* (n=18)
Z'	0.86 ± 0.03 (n = 27)

* CV values shown represent average of DMSO plates and low concentration plates only