Protocol of ESRE HeLa Cell-based Assay for High-throughput Screening

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ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
ATF6 (Recombinant)	HeLa	Human	Cervix	Beta lactamase reporter	Invitrogen	Stress response

QUALITY CONTROL PRECAUTIONS:

1. -Cell culture is maintained by passaging twice a week and should not reach more than 90% confluence
2. -The assay should be performed in black-clear bottom 1536 well plates, so the bottom of the plates should not be touched

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
-DMEM+Glutamax	-Invitrogen	-Invitrogen/10569
-Opti-MEM	-Invitrogen	-Invitrogen/11058
-Dialyzed FBS	-Invitrogen	-Invitrogen/26400
-NEAA	-Invitrogen	-Invitrogen/11140
-Sodium pyruvate	-Invitrogen	-Invitrogen/11360
-HEPES	-Invitrogen	-Invitrogen/15630
-Penn-strep	-Invitrogen	-Invitrogen/15140
-Blasticidin S HCl	-Invitrogen	-Invitrogen/A11139-03
-Recovery Cell Culture Freezing Medium	-Invitrogen	-Invitrogen/12648
-0.05% Trypsin-EDTA	-Invitrogen	-Invitrogen/25300
-17-Allylamino-geldanamycin (17-AAG)	-LC Laboratories	-LC Laboratories/A-6880
-Tetraoctyl ammonium bromide	-Sigma	-Sigma/294136

-LiveBLAzer B/G FRET substrate	-Invitrogen	-Invitrogen/K1028
-CellTiter-Glo One Solution Assay	-Promega	-Promega/G8462
-Black-clear bottom 1536 well plates	-Greiner	-Greiner/789092F
-BioRAPTR FRD dispenser	-Beckman Coulter	-Beckman Coulter
-Multidrop COMBI	-Thermo Electron Corporation	-Thermo Electron Corporation
-Envision Plate Reader	-Perkin Elmer	-Perkin Elmer
-ViewLux Plate Reader	-Perkin Elmer	-Perkin Elmer
-17-AAG or 17-Allylamino-geldanamycin (Agonist control compound)	-LC Laboratories	-LC Laboratories/A-6880

PROCEDURE:

1. Cell handling:
   1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
-DMEM+Glutamax	-90%	-	-90%	-
-Opti-MEM	-	-99.5%	-	-
-Dialyzed FBS	-10%	-0.5%	-10%	-
-NEAA	-0.1mM	-0.1mM	-0.1mM	-
-Sodium pyruvate	-	-1mM	-	-
-HEPES	-25mM	-	-25mM	-
-Penn-strep	-100U/ml-100ug/ml	-100U/ml-100ug/ml	-100U/ml-100ug/ml	-
-Blasticidin S HCl	-5 ug/mL	-	-	-
-Recovery Cell Culture Freezing Medium	-	-	-	-100%

   1.2. Thawing method

      1.2.1 -1ml frozen cells of ESRE bla HeLa 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 in T-75 flask at 2 million cells

   1.3. Propagation method

      1.3.1 -Rinse the cells with DPBS and detach them by using 0.05% Trypsin and centrifuge

1.3.2 -The cells are further passaged at a density of 3 million cells per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in assay medium at a density of 0.25 X 10^6 cells/mL
2.2 -Plate the cells in black-clear bottom 1536 well plate at 1500/well/6uL of assay medium through 8 tip of a plate dispenser (Multi drop)
2.3 -Incubate at 37C for an overnight (18hrs)
2.4 -Transfer 23nL of compounds from the library collection and positive control through Pintool
2.5 -Incubate at 37C for 5hrs
2.6 -Add 1uL of CCF4 dye using a single tip of a plate dispenser (Bioraptr)
2.7 -Incubate at room temperature for 2hrs
2.8 -Read the fluorescence intensity through Envision plate reader
2.9 -Add 4uL of CellTiter-Glo reagent using a single tip of a plate dispenser (Bioraptr)
2.10 -Incubate at room temperature for 30 min
2.11 -Read the luminescence through ViewLux plate reader

3. Assay Performance

EndoR (17-AAG; Agonist control)	**Online Validation Agonist (Mean ± SD)**	**Online Validation Viability (Mean ± SD)**
EC50	0.69 ± 0.05 μM (n = 27)	NA
S/B	2.95 ± 0.08	30.94 ± 2.07
CV (%)*	3.31 ± 0.23 (n = 18)	8.57 ± 1.50 (n = 18)
Z'	0.70 ± 0.05	0.69 ± 0.11

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