Protocol of PPAR-gamma-BLA HEK 293H Cell-based Assay for High-throughput Screening

DOCUMENT: PPAR-gamma-BLA_TOX21_SLP_Version1.0

TITLE: Protocol of PPAR-gamma-BLA HEK 293H Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
PPARgamma: LBD (Recombinent)	HEK 293H	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 is they are more 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+Glutamax	-Invitrogen	-11965
-Phenol Red free DMEM	-Invitrogen	-21063
-Dialyzed FBS	-Invitrogen	-26400
-Charcoal-Stripped FBS	-Invitrogen	-12676-029
-Sodium Pyruvate	-Invitrogen	-11360
-Penn-strep	-Invitrogen	-15140
-NEAA	-Invitrogen	-11140
-HEPES	-Invitrogen	-15630
-Hygromycin	-Invitrogen	-10687-010
-Geneticin	-Invitrogen	-10131-027
-Multidrop	- Thermofisher	-
-BiorapTR dispenser	-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+Glutamax	-90%	-	-90%	-
-Phenol Red free DMEM	-	-99%	-	-
-Dialyzed FBS	-10%	-	-10%	-
-Charcoal-Stripped FBS	-	-1%	-	-
-Penn-strep	-1%	-1%	-1%	-
-Sodium Pyruvate	-1 mM	-	-1 mM	-
-NEAA	-0.1 mM	-	-0.1 mM	-
-HEPES	-25 mM	-	-25 mM	-
-Hygromycin	-100 ug/ml	-	-	-
-Geneticin	-500 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 T175 flask using 30 ml thawing medium

   1.3. Propagation method

      1.3.1 -Detach the cells from the flask using TrypLExpress
      1.3.2 - The cells are re-seeded in T-175 flask at 3-4 million

2. Assay Protocol

   2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing
   2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density

2.3 -Plate the cells in black-clear bottom 1536 well plate at 3000/well/6uL for agonist mode and 3000cells/well/5 ul for antagonist mode through 8 tip Multidrop plate dispenser
2.4 -Incubate for 5 hrs at 37ºC / 99% 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 1 uL of buffer and 1uL of Agonist concentration to respective columns as per plate map for antagonist mode
2.7 -Incubate for 17 hrs at 37ºC / 99% Humidity / 5% CO2
2.8 -Add 1uL of CCF4 (FRET Substarte) dye using a single tip plate dispenser (Bioraptr)
2.9 -Incubate at room temperature for 1 hrs in dark
2.10 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamaze protocol optimized for this cell type
2.11 -Add 3 uL of Cell Titer Glo and Incubate at room temperature for 0.5 hrs in dark for antagonist mode
2.12 -Read on ViewLux protocol optimized for this cell type for antagonist mode

3. Assay Performance

PPARg-bla Agonist (Rosiglitazone)	Online Validation (Mean ± SD)
EC50	0.01 ± 0.002 µM (n = 27)
S/B	3.18 ± 0.07
CV (%)	3.68 ±0.71* (n = 20)
Z'	0.81 ± 0.03

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