Protocol of Shh 3T3 Gli3 Cell-based Assay for High-throughput Screening

DOCUMENT: Shh 3T3 Gli3_TOX21_SLP_Version1.0

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ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Gli1	NIH/3T3	Mouse	Mouse embryo	Luciferase reporter	DMB/OARSA/CFSAN/FDA	Sonic Hedgehog (Shh) pathway

QUALITY CONTROL PRECAUTIONS:

1. Maintain cells below 85% confluence.
2. Assay medium contains only 3% Bovine Calf serum.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vendor/Catalog Number
-DMEM	-Invitrogen	-Invitrogen/11960
-Bovine Calf Serum (BCS)	-ATCC	-ATCC/30-2030
-L-Glutamine	-Invitrogen	-Invitrogen/25030
-Puromycin	-Invitrogen	-Invitrogen/A11138-03
-Penicillin & Streptomycin	-Invitrogen	-Invitrogen/15140
-Recovery Cell culture Freezing Medium	-Invitrogen	-Invitrogen/12648
-0.05% Trypsin-EDTA	-Invitrogen	-Invitrogen/25300
-Cyclopamine	-Enzo Life Sciences	-Enzo Life Sciences/ BML-GR334-0005
-Tetraoctyl ammonium bromide (Positive control for Cytotoxicity)	-Sigma Aldrich	-Sigma Aldrich/294136
- White-solid bottom, Tissue treated 1536-well assay plates	-Greiner Bio-One	-Greiner Bio-One / 789173-F
-MULTIDROP COMBI	-Thermo Electron Corporation	-Thermo Electron Corporation
-BioRAPTR FRD dispenser	-Beckman Coulter	-Beckman Coulter
-ViewLux Plate Reader	-Perkin Elmer	-Perkin Elmer
-Amplite Luciferase reporter gene assay kit	-AAT Bioquest	-AAT Bioquest/12520
-CellTiter-Fluor(TM) Cell Viability Assay	-Promega	-Promega/G6082

-Conditioned Medium	-Supplied by the assay provider	

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
-DMEM	-90%	-97%	-90%	-
- Bovine Calf Serum (BCS)	-10%	-3%	-10%	-
-L-Glutamine	-2 mM	-2 mM	-2 mM	-
-Puromycin	-2 ug/mL	-	-	-
-Penicillin & Streptomycin	-100U/ml & 100ug/ml	-100U/ml & 100ug/ml	-100U/ml & 100ug/ml	-
-Recovery Cell culture Freezing Medium	-	-	-	-100%

1.2. Thawing method

1.2.1 - Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
1.2.2 - Take 1mL of frozen Shh 3T3 Gli3 cells in pre-warmed 9ml of thaw medium and centrifuge.
1.2.3 – Re-suspend the pellet with the thaw medium and cells were seeded in T-225 flask at 3.0 million cells

1.3. Propagation method

1.3.1 -Aspirate medium, rinse twice in DPBS and add 0.05% Trypsin/EDTA (3 mL for a T75 flask) 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 37C and then collect it into a tube for centrifugation.
1.3.3 -The cells can be further passed to the next passage if required.

2. Assay Protocol

2.1 –Harvest cells from the culture flask followed by centrifuging and then re-suspending in assay medium to a density of 0.4 X 10^6 cells/mL.
2.2 –Dispense 2000 cells per well in 5uL of assay medium containing 3% BCS into 1536-well tissue treated white/solid bottom plates using an 8 tip dispenser (Multidrop).
2.3 –Incubate the assay plates for 3-4hr at 37C and 5% CO2.
2.4 –Then transfer the compounds at 23nL from the library collection into 5-48 columns and positive control into 1-4 columns using a Pintool station.
2.5 –Compound transfer is followed by the addition of 1uL of Conditioned medium or assay buffer using two separate tips of a dispenser (Bioraptr).
2.6 –Incubate the assay plates for 24hr at 37C and 5% CO2.
2.7 –After 23hr incubation at 37C, add 1ul of CellTiter-Fluor(TM) cell viability reagent using a single tip of a dispenser (Bioraptr).
2.8 –Incubate the assay plates at 37C for 1hr.
2.9 –Measure the fluorescence intensity using ViewLux plate reader.
2.10 –Then followed by the addition of 4ul of Amplite(TM) luciferase reagent using a single tip of a dispenser (Bioraptr).
2.11 –Incubate the assay plates at room temperature for 30min.

2.12 –Measure the luminescence intensity using (exposure time = 90sec) ViewLux plate reader.