Protocol of Retinol Signaling Pathway C3H10T1/2 Cell-based Assay for High-throughput Screening

DOCUMENT: Retinol Signaling Pathway_TOX21_SLP_Version1.0

TITLE: Protocol of Retinol Signaling Pathway C3H10T1/2 Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Retinoic acid receptor	C3H10T1/2	Mouse	Mouse embryo	Luciferase reporter	OARSA/CFSAN/FDA	Retinol Signaling Pathway (RSP)

QUALITY CONTROL PRECAUTIONS:

1. -Maintain cells below 85% confluence
2. -Fetal Bovine serum used for cell culture and assay purpose is heat inactivated at 56 C for 30min
3. -Extra precautions to be taken for making Retinol as it is photosenitive and moisture absorbant

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
-Eagles Basal Medium: BME	-Invitrogen	-Invitrogen/21010
-Fetal Bovine Serum	-ATCC	-ATCC/30-2020
-L-Glutamine	-Invitrogen	-Invitrogen/25030
-Puromycin	-Invitrogen	-Invitrogen/A11138-03
-Penicillin & Streptomycin	-Invitrogen	-Invitrogen/15140
-Recovery Cell culutre Freezing Medium	-Invitrogen	-Invitrogen/12648
-0.05% Trypsin-EDTA	-Invitrogen	-Invitrogen/25300
-Retinol	-Sigma	-Sigma/95144
-ER50891	-Tocris	-Tocris/3823
-Tetraoctyl ammonium bromide	-Sigma	-Sigma/294136
-1536-well white solid plates	-Greiner Bio-One	-Greiner Bio-One / 789173-F
-MULTIDROP COMBI	-Thermo Electron Corporation	-Thermo Electron Corporation
-BioRAPTR FRD	-Beckman Coulter	-Beckman Coulter
-ViewLux Plate Reader	-Perkin Elmer	-Perkin Elmer
-CellTiter-Glo One Solution Assay	-Promega	-Promega / G8462

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
-Eagles Basal Medium: BME	-90%	-90%	-90%	-
-FBS (Heat inactivated)	-10%	-10%	-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 culutre Freezing Medium	-	-	-	-100%

1.2. Thawing method

1.2.1 -Thaw a vial of cells in 9ml of pre-warmed thaw medium and then centrifuge
1.2.2 -Resuspend the pellet with the thaw medium and seed at 2 million cells per T-75 flask

1.3. Propagation method

1.3.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium
1.3.2 -Passage cells at 1-1.5 million 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 -Dispense 1000 cells/4uL/well into 1536-well tissue treated white/solid bottom plates using a 8 tip dispenser (Multidrop)
2.3 -Incubate the plates for an overnight (20hr) at 37C and 5% CO2
2.4 -Transfer 23nL of compounds from the library collection (5.6nM to 92uM) and positive control through pintool
2.5 -Compound transfer was followed by the addition of 1ul of 1uM (final concentration) Retinol (Retinol made fresh from the powder) or assay buffer using two different tips of a Bioraptr
2.6 -Incubate the plates for 6hr at 37C and 5% CO2
2.7 -Then add 5ul of CellTiter-Glo reagent using a single tip dispense (Bioraptr)
2.8 -Incubate the plates at room temperature for 30min
2.9 -Measure luminescence (exposure time = 1sec) by ViewLux plate reader

3. Assay Performance

RSP	**Online Validation CellTiter-Glo Viability (Antagonist mode) (Mean ± SD)**
IC50	NA
S/B	42.39 ± 1.36

CV (%)*	4.48 ± 0.54 (n = 18)
Z’	0.91 ± 0.02

*CV values shown represent average of all assay plates excluding the top 3 compound concentration plates.