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 |
| -ER 50891 | -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 |
| -Amplite Luciferasse reporter gene assay kit | -AAT Bioquest | -AAT Bioquest/12520 |

**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 -Add 5ul of Amplite(TM) Luciferase reagent using a single tip dispense (Bioraptr)

2.8 -Incubate the plates at room temperature for 30min

2.9 -Measure luminescence (exposure time = 60sec) by ViewLux plate reader

3. Assay Performance

|  |  |
| --- | --- |
| **RSP** | **Online Validation**  **Antagonist**  **(Mean** ± **SD)** |
| IC50 | 0.03 ± 0.01 μM  (n = 27) |
| S/B | 5.48 ± 0.79 |
| CV (%) | 13.15 ± 0.69 ⃰  (n = 18) |
| Z’ | 0.42 ± 0.10 |

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