Protocol of ROR-gamma CHO Cell-based Assay for High-throughput Screening

DOCUMENT: ROR-gamma_TOX21_SLP_Version1.0

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ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Retinoid-related Orphan Receptor gamma	CHO	Human	Chinese Hamster Ovary	Luciferase reporter	Dr. Jetten	ROR pathway

QUALITY CONTROL PRECAUTIONS:

1. -Maintain cells below 85-90% confluence.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
-F12 medium	-Invitrogen	-Invitrogen/11765
-FBS approved to use with Tet-on system	-Clontech	-Clontech/631101
-Penicillin & Streptomycin	-Invitrogen	-Invitrogen/15140
-Recovery Cell culture Freezing Medium	-Invitrogen	-Invitrogen/12648
-0.05% Trypsin-EDTA	-Invitrogen	-Invitrogen/25300
-TO901317 (Antagonist control compound)	-Sigma	-Sigma/T2320
-Doxycycline Hyclate	-Sigma	-Sigma/D9891
-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

-ONE-Glo(TM) Luciferase Assay System	-Promega	-Promega / E6120

PROCEDURE:

1. Cell handling:
   1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
-F12 medium	-90%	-90%	-90%	-
-FBS approved to use with Tet-on system	-10%	-10%	-10%	-
-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 -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 2-3 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 5hrs at 37C and 5% CO2
   2.4 -Transfer 23nL of compounds from the library collection (0.59nM to 92uM) and positive control through Pintool
   2.5 -Incubate the plates for 2hrs at 37C and 5% CO2
   2.6 -Add 1ul of 1.0uM (final concentration) Doxycycline Hyclate in assay buffer using single tip dispense (Bioraptr)
   2.7 -Incubate the plates for 16hrs at 37C and 5% CO2
   2.8 -Then add 5ul of ONE-Glo(TM) Luciferase reagent using a single tip dispense (Bioraptr)
   2.9 -Incubate the plates at room temperature for 30min
   2.10 -Measure luminescence (exposure time = 5 sec) by ViewLux plate reader

3. Assay Performance

RORγ (TO901317; Antagonist control)	**Online Validation Antagonist (Mean ± SD)**
IC50	6.02 ± 0.59 μM (n = 27)
S/B	77.52 ± 10.49
CV (%)*	11.73 ± 0.41 (n = 18)
Z'	0.64 ± 0.04

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