Protocol of TSHR-Hek293 Cell-based Assay for High-throughput Screening

DOCUMENT:　　　　　TSHR-Hek293 _TOX21_SLP

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ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Thyroid Stimulating Hormone Receptor (TSHR), LBD	Hek293	Human	Embryonic kidney cells	Fluorescence	Codex	Gs-coupled TSHR signaling

QALITY CONTROL PRECAUTIONS:
1. Cells should be grown to reach 80 to 90% confluence.
2.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Vender	Catalog Number
DMEM, (high-glucose	Invitrogen	10995
Fetal bovine serum	Invitrogen	26140
Penicillin/Streptomycin	Invitrogen	15140
DPBS	Invitrogen	14190
0.05% Trypsin/EDTA	Invitrogen	25300
Puromycin	Invitrogen	A11138
DMSO	AMRESCO	KD Medical, RGE-3070
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648
TSH	Fitzgerald	30-AT09
Ro20-1724	Sigma	B8279
cAMP dynamic 2	Cisbio	62AM4PEC
White-solid bottom, 1536-well assay plates	Greiner Bio-One	Greiner, 789093
PinTool	Kalypsys	
BioRAPTR™, Microfluidic Workstation	Beckmen	
EnVision plate reader	Perkin Elmer	

PROCEDURE:
1. Cell handling:
   1.1. Medium Required

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
DMEM	90%	90%	90%	-
FBS	10%	10%	10%	-
Penicillin-Streptomycin	100 U/mL-100 µg/mL	100 U/mL-100 µg/mL	100 U/mL-100 µg/mL	-
Puromycin	1 µg/mL	-	-	-

Recovery™ Cell Culture Freezing Medium	-	-	-	100%

  1.2. Thawing method

     1.21 Place 14 mL of pre-warmed thaw medium into a 15 mL of conical tub.

     1.22 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.

     1.23 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.

     1.24 Transfer the vial contents drop-wise into 14 mL of thaw medium in a sterile 15-mL conical tub.

     1.25 Centrifuge cells at 900 rpm for 4 minutes and resuspend in thaw medium.

     1.26 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator.

     1.27 Switch to growth medium at first passage.

  1.3. Propagation method

     1.31 Aspirate medium, rinse once in DPBS, add 0.05% Trypsin/EDTA and swirl to coat the cell evenly.

     1.32 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.

     1.33 Centrifuge cells at 900 rpm for 4 minutes and resuspend in growth medium.

     1.34 Cell should be passage or fed at least twice a week.

2. Assay protocol for agonist mode

  2.1 Harvest cells and resuspend in assay medium.

  2.2 Dispense 800cells/4µL/well into 1536-well tissue treated white-solid-bottom plates using a Multi-drop dispenser.

  2.3 After the cells were incubated at 37°C for 18 hours, 23 nL of compounds dissolved in DMSO, positive controls or DMSO were transferred to the assay plate by a PinTool resulting in a 217-fold dilution.

  2.4 Followed by 1 µl assay medium containing Ro 20-1724 (final concentration is 25 µM) and TSH (final concentration is 1.5 ng/mL) to each well.

  2.5 Incubate the plates for 0.5 hours at room temperature.

  2.6 Add 2.5 µL of cAMP-d2, and then add 2.5 µl of anti cAMP-Cryptate to each well using a BioRAPTR dispenser.

  2.7 After one hour incubation at room temperature, measure fluorescence intensity at 665 and 620 nm emission and 340 nm excitation by an Envision detector. Data is expressed as the ratio of 665nm/620nm emissions.

3. Assay Performance

TSHR-cAMP Antgonist	Online Validation (Mean ± SD)
EC50	N/A
S/B	2.55± 0.11 (n = 23)
CV (%)	5.71 ± 1.00 (n=23)
Z'	0.53 ± 0.10 (n = 23)