Protocol of HEK293-TRHR Cell-based Assay for High-throughput Screening

DOCUMENT: HEK293-TRHR_TOX21_SLP_Version1.0

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ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
TRHR	Hek293-TRHR	Human	Embryonic kidney	Fluorescence	Codex	Gs-coupled TRHR signaling

QUALITY CONTROL PRECAUTIONS:

1. The cells should not be grown more than 80-85% confluence
2. Do not leave cells in Trypsin for more than 5 min at RT

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM, high-glucose	Invitrogen	11995
FBS	Invitrogen	26140
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140
Puromycin	Invitrogen	A11138
Recovery Cell Culture Freezing Medium	Invitrogen	12648
Cal-520 calcium assay kit	AAT Bioquest	36400
TRH	Sigma	P1319
FDSS 7000EX kinetic plate reader	FDSS 7000EX	-
Multidrop	Thermofisher	-
BioRAPTR	Beckman Coulter	-

PROCEDURE:

1. Cell handling:
1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
DMEM, high glucose	90%	90%	90%	-
FBS	10%	10%	10%	-
Penicillin/Streptomycin (antibiotic)	1%	1%	1%	-
Puromycin	1ug/mL	1ug/mL	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a 15 mL conical tube
1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath
1.2.3 Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO
1.2.4 Discard the supernatant and reconstitute the pellet using pre-warmed thaw medium
1.2.5 Transfer the cells to T75 flask

1.3. Propagation method

1.3.1 Detach the cells from the flask using Trypsin-EDTA (0.05%)
1.3.2 The cells in growth medium are re-seeded in T225 flask

2. Assay Protocol

2.1 Harvest cells and re-suspend in assay medium and adjust the required cell density
2.2 Dispense 1500 cells/4 µL/well into 1536 well tissue treated black-clear-bottom plates using a Multidrop dispenser
2.3 Incubate the plates at a 37ºC, 5% CO2 incubator for 18 h
2.4 Add 4 µL of Loading Buffer
2.5 Incubate at 37ºC, 5% CO2 for 2 h
2.6 Incubate at RT for 15 min
2.7 Transfer 23nL of compounds and positive control to the assay plate by a pin tool.
2.8 Read the fluorescence intensity in FDSS 7000EX kinetic plate reader with a filter set of Ex/Em=480/540 for 3 min at 1 sec intervals
2.9 For antagonist mode, add 1 µL of TRH at 0.1 nM (final) to each well except 16 wells in column 4 after 5 min incubation at RT.
3.0 Read the fluorescence intensity in FDSS 7000EX kinetic plate reader with a filter set of Ex/Em=480/540 for 3 min at 1 sec intervals

3. Assay Performance

TRHR-agonist **(TSH)**	Online Validation (Mean ± SD)

AC50 (nM)	0.05±0.04
S/B	48.15 ± 12.93
CV (%)	25.90 ± 8.17*
Z’	0.79 ± 0.06
N	27

TRHR-antagonist **(N/A)**	Online Validation (Mean ± SD)
AC50 (nM)	N/A
S/B	62.58 ± 11.05
CV (%)	6.70 ± 1.32*
Z’	0.81 ± 0.05
N	27

*CV values shown represent average of DMSO plates and low concentration plates only (n=18).