Protocol of CHRM1-CHO Cell-based Assay for High-throughput Screening

DOCUMENT: *CHRM1-CHO* _TOX21_SLP

TITLE: Protocol of *CHRM1-CHO* cell-based assay for high-throughput screening.

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
muscarinic acetylcholine receptor M1 (CHRM1)	CHO	Hamster	Ovary	Fluorescence	Codex	Gq-coupled CHRM1 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/F12	Sigma	D8437
Fetal bovine serum	Invitrogen	26140
Penicillin/Streptomycin	Invitrogen	15140
DPBS	Invitrogen	14190
0.05% Trypsin/EDTA	Invitrogen	25300
Puromycin	Invitrogen	A11138
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648
Acetylcholine	Sigma	
Atropine	Sigma	
Cal-520 calcium assay kit	AAT Bioquest	
Black-clear bottom, 1536-well assay plates	Greiner Bio-One	
BioRAPTR™, Microfluidic Workstation	Beckmen	
FDSS 7000EX kinetic plate reader	Hamamatsu	

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	10 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 antagonist mode

2.1 Harvest cells and resuspend in assay medium.

2.2 Dispense 1000cells/3µL/well into 1536-well tissue treated black-clear-bottom plates using a Multi-drop dispenser.

2.3 After the cells were incubated at 37°C for 18 hours, add 3µl of Loading Dye to each well.

2.4 Incubate the plates for 1 h at 37°C.

2.5 Incubate the plates for 15 m at room temperature

2.6 Add 23 nL of compounds dissolved in DMSO, positive controls or DMSO by a PinTool.

2.7 Read the fluorescence intensity in FDSS 7000EX kinetic plate reader with a filter set of Ex/Em=480/540 for 2 min at 1 sec intervals

2.8 For antagonist mode, incubate the plates for 5 m at room temperature after step 2.6

2.9 Add 1.5 µL of Acetylcholine at 50 nM to each well

2.10 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

CHRM-CHO agonist	Online Validation (Mean ± SD)
EC50 (nM)	17.23 ± 3.70
S/B	56.45 ± 17.32
CV (%)	19.27 ± 6.51
Z’	0.83 ± 0.03

CHRM-CHO antagonist	Online Validation (Mean ± SD)

EC50 (nM)	3.10 ± 0.75
S/B	36.89 ± 7.36
CV (%)	7.06 ± 2.27
Z’	0.80 ± 0.06