Protocol of CAR1 HepG2 Cell-based Assay for High-throughput Screening

DOCUMENT: CAR1_TOX21_SLP_Version1.0

TITLE: Protocol of CAR1 HepG2 Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
CAR1 (full-length receptor)	HepG2	Human	Hepatocellular carcinoma	Luciferase	Dr. Hongbing Wang and Dr. Caitlin Lynch	-

QUALITY CONTROL PRECAUTIONS:

1. Cells should be grown and passaged in a collagen coated flask.
2. ONEglo should be used over other luminescent reagents. We did multiple test runs and found ONEglo to have higher quality.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Life Technologies	10566
Recovery Cell Culture Freezing Medium	Life Technologies	12648
HyClone® FBS	Thermo Scientific	SH30071.03
Pen-Strep	Life Technologies	15140
Blasticidin	Life Technologies	A11139-03
Geneticin	Life Technologies	10131-027
Trypsin-EDTA (0.25%)	Life Technologies	25200-056
ONE-Glo Luciferase Buffer	Promega	E6051
Multidrop	Thermofisher	-
BiorapTR	Beckman Coulter	-
ViewLux Plate Reader	Perkin Elmer	-

PROCEDURE:

1. Cell handling:
   1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	90%	90%	-
HyClone® FBS	10%	10%	10%	-
Pen-strep	1%	1%	1%	-
Blasticidin	5 µg/mL	5 µg/mL	5 µg/mL	-
Geneticin	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	-

   1.2. Thawing method

      1.2.1. Place 9 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 with gentle agitation for 1-2 minutes.
      1.2.3. Mix the entire content of the vial to the 9 mL of pre-warmed medium and centrifuge to remove DMSO
      1.2.4. Discard the supernatant and reconstitute the pellet using 10 mL of pre-warmed media.
      1.2.5. Transfer the necessary amount of reconstituted cells to a T-75 collagen-coated flask using 30 mL thawing medium

   1.3. Propagation method

      1.3.1. Detach the cells from the flask using Trypsin-EDTA (0.25%)
      1.3.2. The cells are re-seeded in T-75 flask at 2.5 - 4 million

2. Assay Protocol

   2.1. Spin down the cells after rinsing the cells with DPBS and trypsinizing
   2.2. Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density
   2.3. Plate the cells in black-clear bottom 1536 well plate at 2500 cells/well/4µL utilizing an 8 tip Multidrop plate dispenser
   2.4. Incubate for 4 hrs at 37ºC / 99% Humidity / 5% CO_2
   2.5. Transfer 23 nL of compounds from the library collection and positive control to the assay plates through pintool
   2.6. Add 1 µL of CITCO (50 nM = final concentration) to every column
   2.7. Incubate for 23 hrs at 37ºC / 99% Humidity / 5% CO_2
   2.8. Add 1 µL of CTF dye using a single tip plate dispenser (Bioraptr)
   2.9. Incubate at 37ºC / 99% Humidity / 5% CO_2 for 1 hr
   2.10. Read the fluorescence intensity through ViewLux plate reader using CTF protocol
   2.11. Add 4 µL of ONE-glo reagent and incubate at room temperature for 0.5 hrs
   2.12. Read on ViewLux luminescence protocol optimized for this cell type

3. Assay Performance

HepG2-CYP2B6-CAR	Online Validation Antagonist mode (mean ±SD)	Online Validation Antagonist Viability (mean ±SD)
CV (%)	4.74 ± 1.25	5.56 ± 0.64
B/I	3.48 ± 0.26	4.34 ± 0.15
Z	0.74 ± 0.07	0.73 ± 0.03
IC_{50} (µM)	1.08 ± 0.30	