

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 REFERENCES:

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 37°C / 99% Humidity / 5% CO₂
- 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 PK11195 (0.75 µM = final concentration) to every column
- 2.7. Incubate for 23 hrs at 37°C / 99% Humidity / 5% CO₂
- 2.8. Add 1 µL of CTF dye using a single tip plate dispenser (Bioraptr)
- 2.9. Incubate at 37°C / 99% Humidity / 5% CO₂ 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 Agonist (mean \pm SD)	Online Validation Viability (mean \pm SD)
CV (%)	7.74 \pm 1.91	4.08 \pm 0.47
S/B	3.96 \pm 0.20	4.18 \pm 0.08
Z	0.66 \pm 0.05	0.80 \pm 0.02
EC ₅₀ (μ M)	0.45 \pm 0.06	