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

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| **DOCUMENT:** |  | CAR1\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of CAR1 HepG2 Cell-based Assay for High-throughput Screening |

**ASSAY RFERENCES:**

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| 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:**

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| 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% 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 37ºC / 99% Humidity / 5% CO2

2.8. Add 1 µL of CTF dye using a single tip plate dispenser (Bioraptr)

2.9. Incubate at 37ºC / 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

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| **HepG2-CYP2B6-CAR** | **Online Validation Agonist**  **(mean ±SD)** | **Online Validation Viability**  **(mean ±SD)** |
| CV (%) | 7.74 ±1.91 | 4.08 ±0.47 |
| S/B | 3.96 ±0.20 | 4.18 ±0.08 |
| Z | 0.66 ±0.05 | 0.80 ±0.02 |
| EC50 (µM) | 0.45 ± 0.06 |  |

3. Assay Performance