Protocol of ARE-BLA HepG2 Cell-based Assay for High-throughput Screening

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| **DOCUMENT:** |  | ARE-BLA\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of ARE-BLA 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 |
| Nrf2/ARE  (Recombinant) | HepG2 | Human | Hepatocellular carcinoma | Beta-lactamase | Invitrogen | Stress response |

**QUALITY CONTROL PRECAUTIONS:**

1.  Cells should be grown to reach 60 to 75% confluence

2.  Handle the 1536-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate

**MATERIALS and INSTRUMENTS:**

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| Supplies/Medium/Reagent | Manufacturer | Vender/Catalog Number |
| DMEM with GlutaMAX | Invitrogen | 10569 |
| Fetal bovine serum, dialyzed | Invitrogen | 26400 |
| Nonessential amino acids (NEAA) | Invitrogen | 11140 |
| Penicillin/Streptomycin (antibiotic) | Invitrogen | 15140 |
| DPBS | Invitrogen | 14190 |
| HEPES (1 M, pH 7.3) | Invitrogen | 15630 |
| 0.25% Trypsin/EDTA | Invitrogen | 25300 |
| Blasticidin (antibiotic) | Invitrogen | R210 |
| LiveBLAzer B/G FRET Loading Kit (Solution A, B and C) | Invitrogen | K1030 |
| Solution D | Invitrogen | K1157 |
| β-Naphthoflavone | Sigma | 70415 |
| Blackwall, clear-bottom, 1536-well assay plates | Greiner BioOne | 789092-F |
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**PROCEDURE:**

1. Cell handling:

1.1. Media Required:

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| --- | --- | --- | --- | --- |
| Component | Growth Medium | Assay Medium | Thaw Medium | Freezing Medium |
| DMEM with GlutaMAX | 90% | 99% | 90% | - |
| Dialyzed FBS | 10% | 1% | 10% | - |
| NEAA | 0.1 mM | 0.1 mM | 0.1 mM | - |
| HEPES (pH 7.3) | 25 mM | 25 mM | 25 mM | - |
| Penicillin/Streptomycin | 100U/mL/100µg/mL | 100U/mL/100µg/mL | 100U/mL/100µg/mL | - |
| Blasticidin (antibiotic) | 5 ug/mL | - | - | - |
| Recovery Cell freezing medium | - | - | - | 100% |

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a T75 flask

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. Do not submerge vial in water.

1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.

1.2.4 Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tub.

1.2.5 Centrifuge cells at 900 rpm for 4 minutes and resuspend in thaw medium.

1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator.

1.2.7 Switch to growth medium at first passage.

1.3. Propagation method

1.3.1 Aspirate medium, rinse once in DPBS, add 0.25% Trypsin/EDTA and swirl to coat the cell evenly.

1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.

1.3.3 Centrifuge cells at 900 rpm for 4 minutes and resuspend in growth medium.

1.3.4 Cell should be passage at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture in growth medium and resuspend in assay medium.

2.2 Dispense 2000 cells/5µL/well into 1536-well tissue treated black/clear bottom plates using a BioRAPTR dispenser.

2.3 After the cells were incubated at 37°C for 5 hours, 23 nL of positive controls or compounds were transferred to the assay plate by a PinTool resulting in a 217-fold dilution. The final compound concentration in the 5 µl assay volume ranged from 1.2 nM to 92 µM in 15 concentrations.

2.4 Incubate the plates for 16 hours at 37°C.

2.5 Add 1 µL of 6X LiveBLAzer-FRET B/G (CCF4-AM) Substrate Mixture to each well using a BioRAPTR dispenser and incubate the plate at room temperature for 2 hours.

2.6 Measure fluorescence intensity at 460 and 530 nm emission and 405 nm excitation by an Envision detector. Data is expressed as the ratio of 460nm/530nm emissions.

3. Assay Performance

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| ARE-bla Agonist  (β-Naphthoflavone) | Online Validation  (Mean ± SD) |
| EC50 | 1.47 ± 0.19 μM  (n = 27) |
| S/B | 3.08 ± 0.26  (n = 27) |
| CV (%) | 10.63 ± 2.12\*  (n=18) |
| Z’ | 0.86 ± 0.03  (n = 27) |

\* CV values shown represent average of DMSO plates and low concentration plates only