Protocol of FXR-BLA HEK 293T Cell-based Assay for High-throughput Screening

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| **DOCUMENT:** |  | FXR-BLA\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of FXR-BLA HEK 293T 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 |
| Farnesoid X receptor: LBD  (Recombinant) | HEK 293T | Human | Embryonic kidney | Beta-lactamase reporter | Invitrogen | NR signaling |

**QUALITY CONTROL PRECAUTIONS:**

1. Cells should be grown to reach 80 to 90% confluence

**MATERIALS and INSTRUMENTS:**

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| Supplies/Medium/Reagent | Manufacturer | Vender/Catalog Number |
| DMEM, with GlutaMAX | Invitrogen | 10569 |
| DMEM, phenol red-free | Invitrogen | 21063 |
| Fetal bovine serum, dialyzed | Invitrogen | 26400 |
| Nonessential amino acids (NEAA) | Invitrogen | 11140 |
| Penicillin/Streptomycin (antibiotic) | Invitrogen | 15140 |
| DPBS | Invitrogen | 14190 |
| Sodium pyruvate | Invitrogen | 11360 |
| HEPES (1 M, pH 7.3) | Invitrogen | 15630 |
| 0.05% Trypsin/EDTA | Invitrogen | 25300 |
| Hygromycin (antibiotic) | Invitrogen | 10687 |
| Zeocin (antibiotic) | Invitrogen | R25001 |
| LiveBLAzer-FRET B/G Loading Kit: Solution A, B and C | Invitrogen | K1030 |
| Recovery Cell Culture Freezing Medium | Invitrogen | 12648 |
| Fetal bovine serum (FBS), charcoal stripped | Invitrogen | 12676-011 |
| Chenodeoxycholic acid (CDCA) | Sigma | C9377 |
| Black, clear-bottom, 1536-well assay plates | Greiner BioOne | 789092-F |
| PinTool | Kalypsys | - |
| BioRAPTR, Microfluidic Workstation | Beckmen | - |
| EnVision plate reader | Perkin Elmer | - |
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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% | - | 90% | - |
| DMEM, phenol red-free | - | 98% | - | - |
| Dialyzed FBS | 10% | - | 10% | - |
| Charcoal stripped FBS | - | 2% | - | - |
| Sodium pyruvate | - | 1mM | - | - |
| NEAA | 0.1 mM | 0.1mM | 0.1 mM | - |
| HEPES (pH 7.3) | 25 mM | - | 25 mM | - |
| Penicillin/Streptomycin | 100U/mL/100µg/mL | 100U/mL/100µg/mL | 100U/mL/100µg/mL | - |
| Hygromycin (antibiotic) | 100 μg/mL | - | - | - |
| Zeocin (antibiotic) | 100 μg/mL | - | - | - |
| Recovery Cell Culture Freezing Medium | - | - | - | 100% |

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a 15 mL of conical tub.

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 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 14 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 0.05% 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 5000 cells/5µL/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 5 hours, 23 nL of control or compounds dissolved in DMSO were transferred to the assay plate by a PinTool resulting in a 217-fold dilution.

2.4 Add 1 uL of agonist (CDCA) at 300 µM in assay medium to the column 1-2 and column 5-48. Add 1 uL of assay medium to the column 3-4.

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

2.6 Add 1 µL of 6X LiveBLAzer-FRET B/G (CCF4-AM) Substrate Mixture to each well using a BioRAPTR dispenser.

2.7 After two hours incubation at room temperature, 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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| FXR-bla-Antagonist  (n/a) | Online Validation  (Mean ± SD) |
| IC50 | n/a |
| S/B | 1.96 ± 0.05  (n=27) |
| CV (%) | 2.32 ± 0.13\*  (n=18) |
| Z’ | 0.87 ± 0.01  (n=27) |

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