Protocol of H2AX CHO-K1 Cell-based Assay for High-throughput Screening

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| **DOCUMENT:** |  | H2AX\_TOX21\_SLP\_Version1.0 |
| **TITLE:** |  | Protocol of H2AX CHO-K1 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 |
| histone-H2AX | CHO-K1 | Hamster | Ovaries | Homogeneous time resolved fluorescence (HTRF) | Cisbio US Inc. | DNA damage |

**QUALITY CONTROL PRECAUTIONS:**

1. -Cells thawed from frozen vials and culture for 2 days have been used for assay purposes

**MATERIALS and INSTRUMENTS:**

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| --- | --- | --- |
| Supplies/Medium/Reagent | Manufacturer | Vender/Catalog Number |
| -F-12K Medium | -ATCC | -ATCC/30-2004 |
| -Fetal Bovine Serum | -Hyclone Laboratories | -Hyclone/SH30071.03 |
| -Penn-strep | -Invitrogen | -Invitrogen/15140 |
| -Recovery Cell Culture Freezing Medium | -Invitrogen | -Invitrogen/12648 |
| -0.25% Trypsin-EDTA | -Invitrogen | -Invitrogen/25200 |
| -HTRF Phospho-H2A.X (S139) Assay, 10,000 tests | - CisBio | -CisBio |
| -White solid bottom 1536 well plates | -Greiner | -Greiner/789173F |
| -BioRAPTR FRD dispenser | -Beckman Coulter | -Beckman Coulter |
| -Multidrop COMBI | -Thermo Electron Corporation | -Thermo Electron Corporation |
| -Envision Plate Reader | -Perkin Elmer | -Perkin Elmer |

**PROCEDURE:**

1. Cell handling:

1.1. Media Required:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Component | Growth Medium | Assay Medium | Thaw Medium | Freezing Medium |
| -F-12K Medium | -90% | -90% | -90% | - |
| -Fetal Bovine Serum | -10% | -10% | -10% | - |
| -Penn-strep | -100U/ml-100ug/ml | -100U/ml-100ug/ml | -100U/ml-100ug/ml | - |
| -Recovery Cell Culture Freezing Medium | - | - | - | -100% |

1.2. Thawing method

1.2.1 -1ml frozen cells of CHO-K1 were taken in to pre-warmed 9ml of thaw medium for centrifuging.

1.2.2 -The cells were seeded in a five-layer flask at a density of 12 million cells.

1.3. Propagation method

1.3.1 -Rinse the cells with DPBS and detach them by using 0.25% Trypsin and centrifuge

1.3.2 -The cells are further passaged at a density of 2.5 million cells per T-225 flask or 12 million cells per five-layer flask.

2. Assay Protocol

2.1 -Harvest cells from the culture and centrifuge and resuspend in assay medium at 0.33X10^6 cells/mL

2.2 -Dispense cells at 1000 per well in 3uL of assay medium into white/solid bottom 1536 well plates using Multidrop COMBI

2.3 -Incubate at 37C for an overnight (18-20 hrs)

2.4 -Transfer 23nL of compounds from the library collection and positive control through pintool

2.5 -Incubate at 37C for 3 hrs

2.6 -Add 1uL of lysis buffer using a single tip of a plate dispenser (Bioraptr)

2.7 -Lysis Buffer is made by diluting 4 fold lysis buffer stock solution with distilled water and diluting 100 fold blocking reagent # 3 in the prepared diluted lysis buffer

2.8 -Centrifuge the assay plates at 1000 rpm for 10 sec

2.9 -Incubate at room temperature for 30 min

2.10 -Add 1uL of antibody detection buffer using a single tip of a plate dispenser (Bioraptr)

2.11 -Antibody detection buffer is made by mixing 1:1 volume mix of Solutions A (Anti H2A.X-d2) and B (Anti pH2A.X (S139)-K), both at 20 fold dilution with detection buffer

2.12 -Incubate at room temperature for 24 hrs

2.13 -Read TR-FRET using Envision plate reader at excitation: 320nm and emissions at 620 and 665nm

3. Assay Performance

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| **pH2AX** | **Online Validation**  **Agonist (3h incubation)**  **(Mean ± SD)** |
| EC50 | 0.57 ± 0.22 μM  (n = 27) |
| S/B | 2.30 ± 0.14 |
| CV (%) | 6.31 ± 0.36 ⃰  (n = 24) |
| Z’ | 0.51 ± 0.07 |

⃰ CV values shown represent average of all assay plates excluding the top concentration plates.