

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

DOCUMENT: H2AX_TOX21_SLP_Version1.0

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

ASSAY REFERENCES:

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:

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%
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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 0.33×10^6 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

pH2AX	Online Validation Agonist (3h incubation) (Mean \pm SD)
EC50	$0.57 \pm 0.22 \mu\text{M}$ (n = 27)
S/B	2.30 ± 0.14
CV (%)	$6.31 \pm 0.36^*$ (n = 24)
Z'	0.51 ± 0.07

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