Protocol of KS_ARE HaCaT human keratinocytes Cell-based Assay for High-throughput Screening (Agonist mode)

DOCUMENT: KS_ARE_TOX21_SLP_Version1.0

TITLE: Protocol of KS_ARE HaCaT human keratinocytes Cell-based Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
Nrf2/ARE	HaCAT	Human	Skin keratinocytes cells	Luciferase reporter	Givaudan, Switzerland	Stress response

QUALITY CONTROL PRECAUTIONS:

1. The cells should not be grown more than 80-85% confluence
2. The cell performance is affected if they are too confluent
3. Do not leave cells in Trypsin for more than 5 min at RT
4. Spin plate for 5 seconds after One-Glo added

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Gibco	10567-014
FBS	HyClone	SH30071.03, heat inactivated
Geneticin (G418)	Gibco	10131-035
Pen-Strep	Invitrogen	15140
0.05% Trypsin-EDTA	Invitrogen	25300-054
tert-Butylhydroquinone	Sigma	112941
Multidrop	-Thermofisher	-
BiorapTR	Beckman Coulter	-
CellTiter- Fluor	Promega	G6080/1/2
Viewlux Plate Reader	Perkin Elmer	-
One-Glo	Promega	E606C/E6051

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%	98%	90%	-
FBS	10%	1%	10%	-
Penn-strep	1%	1%		-
G418	500µg/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 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. Do not submerge vial in water.
1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO
1.2.4 -Discard the supernatant and transfer the precipitated cells to T225 flask using 45 ml thawing medium

1.3. Propagation method

1.3.1 -Detach the cells from the flask using 0.05% Trypsin-EDTA
1.3.2 -The cells are re-seeded in T-225 flask at 2.5 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing
2.2 –Re-suspend the pellet with assay medium followed by filtering through cell strainer and adjust to the required cell density
2.3 -Plate the cells in white-solid bottom 1536 well plate at 1500/well/5µl for agonist mode through 8 tip Multidrop plate dispenser
2.4 -Incubate for 6hrs at 37ºC / 95% Humidity / 5% CO2
2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool
2.6 -Incubate for 23hrs at 37ºC / 95% Humidity / 5% CO2
2.7 -Add 1µl of CellTiter- Fluor using a single tip plate dispenser (Bioraptr)
2.8 -Incubate at 37ºC / 95% Humidity / 5% CO2 for 1hr
2.9 -Read the fluorescence intensity through Viewlux plate reader using protocol optimized for this cell type
2.10 -Add 4µl of One-Glo, spin plate for 5sec and incubate at room temperature for 30min
2.11 -Read luminescence intensity through ViewLux plate reader using protocol optimized for this cell type

3. Assay Performance

3.1 Performance of online validation:

KS-ARE Agonist (tBHQ)	Online Validation (Mean ± SD)	Online Validation Viability (Mean ± SD)
EC50	85.05 ± 15.63 nM (n =27)	N/A
S/B	3.27 ± 0.17	8.89 ± 0.68
CV (%)	7.98 ± 1.19 (n = 24)	3.33 ± 1.33 (n = 24)
Z'	0.41 ± 0.12	0.87 ± 0.02

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

3.2 Performance of online screening:

Assay	Active match	Inactive match	Inconclusive	Mismatch	AC50 fold change	Score
tox21-ks-are-p1	11.60%	75.74%	12.30%	0.35%	1.40	85.93
tox21-ks-are-p1_viability	9.32%	84.68%	5.89%	0.11%	1.28	97.21