Use of Assay Ready HaCaT Cells for Cytotoxicty Testing

The epithelium serves as a gatekeeper of the human body and protects it from adverse impacts of the environment. Human skin epithelial cells (keratinocytes) are therefore a well accepted model to asses the skin irritating and cytotoxic potential of chemicals. HaCaT cells, a spontaneous immortalized cell line of human keratinocytes, can be cryopreserved in an assay ready state to be used instantly for cytotoxicity testing without any prior cultivation. This eliminates a big factor which generates inter laboratory variability and enables a reproducible use of these cells for routine safety testing.

introduction

All chemicals to which humans may be exposed need to be tested for their toxicity in order to counter the risk to handle them and to take appropriate safety measures. Since 2007, REACh regulates the registration and evaluation of chemicals in general while other substances, e.g. pharmaceutical or cosmetic ingredients, food additives, or biocides are evaluated according to the respective industry guidelines. Whichever guideline is relevant, cell based *in vitro* models measuring the

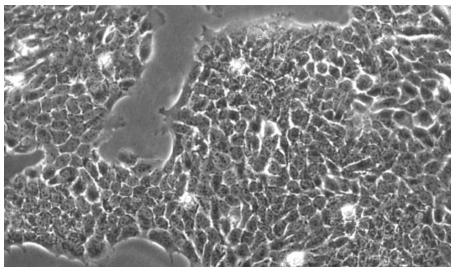

Fig.1: assay ready HaCaT cells 24 hours after plating.

viability of cells after a certain exposure time usually provide a solid estimate of the toxic potential of a substance.

Depending on the way of exposure, different cell types are used. HaCaT, a spontaneous immortalized cell line of human keratinocytes, is a well accepted model widely used for different applications including toxicity testing [1]. The cell line has been developed in 1988 by Boukamp et al [2] from a skin biopsy of a 62-year old male.

A reproducible and routine use of cells requires proper and well standardized cell culture procedures. To minimize cell culture related variances assay ready cells proved to be a valuable tool. Assay ready cells are cryopreserved at a highly viable and functional state and can be used in assays like a typical reagent without any prior cultivation. Here the reliability and reproducibility of assay ready HaCaT cells in cytotoxicity testing have been investigated.

material and methods

cultivation of HaCaT cells

HaCaT cells have been obtained from CLS Cell Lines Service (Heidelberg, Germany), at passage 31 and were cultivated in DMEM (Sigma Aldrich) with 10% FBS (PAN Biotech) and 5mM L-Glutamine (Sigma Aldrich). For further expansion sub-confluent cultures were detached with Accutase (Sigma Aldrich) and plated at a density of 2E4 cells/cm^2 in CellStacks (Corning). Cells were cultivated at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of assay ready frozen cells

Expanded cells of passage 50 were harvested from CellStacks using Accutase. The detached cells were washed and resuspended in a serum free cryopreservation medium containing 10% DMSO. An XSD-Biofill (FuidX) was used to dispense the cells automatically into 2ml cryovials (Nunc). Finally the cells were cryopreserved in a Cryomed 7452 controlled rate freezer (Thermo Fisher) at a cooling rate of 1°C per minute. The assay ready frozen cells were stored in vapor phase of liquid nitrogen.

determination of viable cell count

A vial of assay ready HaCaT cells was thawed in a water bath at 37°C and a sample of the cells was analyzed in a CASY Model TT (Roche Innovatis AG) to determine the actual cell count, the viability, the amount of debris and the grade of aggregation.

cytotoxcitiy testing

Assay ready HaCaT cells were thawed, washed once in recovery buffer and dispensed into 96-well plates at 6E4 cells/well.

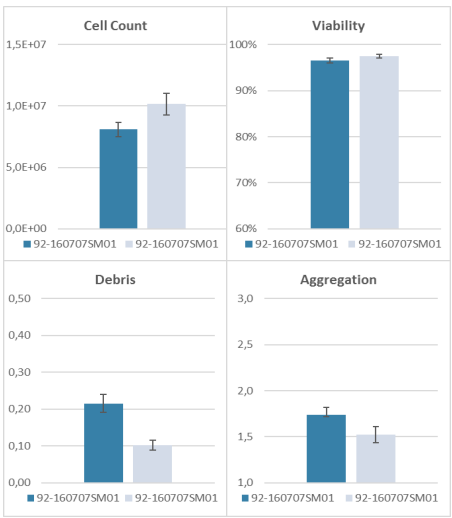

Fig.2: viability parameters of assay ready HaCaT cells

Serial dilutions of test chemicals were applied to the cells immediately thereafter and incubated with the cells for 24 hours. Toxicity was quantified by measuring the metabolic activity of the surviving cells. Resazurin (Sigma Aldrich) which is converted by viable cells into a pink colored and fluorescent dye was added. Fluorescence was measured after 4 hours

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from top in a Safire-2 plate reader (Tecan) at $540_{Em}/590_{Ex}$.

results

viability parameters of assay ready HaCaT

Two independent batches of assay ready frozen HaCaT with 10 million cells per vial, have been prepared each expanded form a separate vial of the Master Cell Bank. 5% of each batch were thawed to determine cell count and viability parameters (Fig. 2). The cell count of batch 92-160707SM01 was about 20% below the targeted 1E7 cells, probably due to a counting error at harvest. However, the lots were very homogenous showing a variation from vial to vial of only 7% and 9%, respectively. The viability of the cells was above 95% for both lots directly after thawing as well a 24 hours after plating. The amount of cell debris, which is a very valuable indicator for cell death as well, did not show significantly elevated levels compared to a continuous grown culture (< 25%). Also the grade of aggregation was below 2 which indicates that frozen aliquots contain mostly single cells and hardly any aggregates.

When plated, assay ready HaCaT display the typical morphology of endothelial cells. Almost no non-attached or dead cells were visible (Fig. 1). Cells seeded at 5E4 cells/cm^2 grew to about 80% within 24 hours.

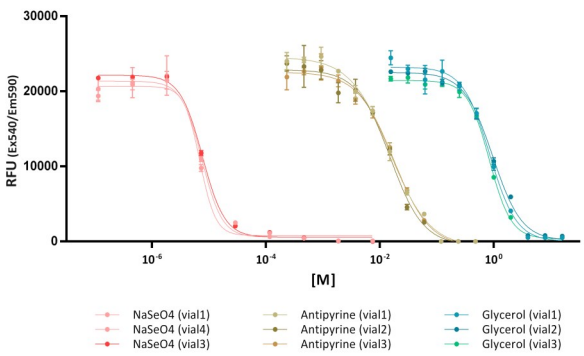

Fig.3: Dose dependent toxicity of Glycerol, Antipyrin, $NaSeO_4$.

sensitivity to cytotoxic chemicals

Assay ready HaCaT cells were used instantly for cytotoxicity testing. Three independent vials of a batch were thawed and a quantity of nominal 60.000 cells were seeded into each well of a 96-well plate. This means the cells have not been counted again but a defined volume equivalent was used. The cells were challenged with reference chemicals immediately after thawing without any prior cultivation. Serial dilutions of Glycerol, Antipyrine, and Sodium Selenite, were applied in triplicates to the cells. The grade of survival was determined by Resazurin and depicted in sigmoidal dose response curves using Graph Pad Prism software (Fig. 3). All vials of assay ready HaCaT generated a maximal signal (Span) above 20,000 RFU (+/- 4%) confirming the viability of th cells and the homogeneity of the lot (Tab.1). The dose response curves generated with cells from individual vials have an almost identical shape and showed a very good parallelism (Fig. 3). The IC50 values determined for the three chemicals were in the expected range for HaCaT cells with a very low variance from vial to vial between 4.3 % and 7.1 %. The data quality was very robust delivering a Z' between 8.7 and 8.9 for all assays (Tab. 1)..

	IC_{50} [M]	Slope	Span (S-B)	Z'
Glycerol	0.88 (+/- 4.3 %)	-2.60 (+/- 15.1%)	20.745 (+/- 3.4%)	0.90
Antipyrine	1.59E-2 (+/- 7.1 %)	-1.37 (+/- 8.6%)	24.003 (+/- 3.9%	0.87
$NaSeO_4$	7.39E-6 (+/- 6.6 %)	-1.94 (+/- 12.1%)	22.248 (+/- 3.9 %)	0.88

Tab.1: characteristics of toxicity dose response curves.

discussion

assay ready HaCaT cells which are cryopreserved at a high viability and quality provide a very robust and reproducible tool for cytotoxicity testing. Although the cells are not supposed to be cultivated or even let adhere before the samples were added, they generate a strong viability signal when incubated with non-toxic concentrations of the chemical but display the expected sensitivity to toxic concentrations. Different vials of assay ready HaCaT cells not only provide identical IC50 value for each of the tested reference compounds but also provide an almost identical maximal signal proving the high robustness and reproducibility of the assay ready cells. Because assay ready cells are prepared and validated in large homogenous batches, variabilities caused by cell handling are eliminated.

literature

[1] Udensi UK, Graham-Evans BE, Rogers CS, Isokpehi RD. Cytotoxicity patterns of arsenic trioxide exposure on HaCaT keratinocytes. Clin Cosmet Investig Dermatol. 2011; 4:183-90.

[2] Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line, J Cell Biol. 1988 Mar;106(3):761-71. Journal of Cell Biology 106 (3): 761–771.

related products

instaCELL® cytotoxicty assay kit (HaCaT)[1]

validated kit with recovery buffer, media, positive control, Resazurin viability dye, 96-well assay plates, and assay ready cryopreserved HaCaT cells.
- 5x 96-well (CatN° SF060-05)

arc - assay ready cells (HaCaT)[1]

assay ready cryopreserved HaCaT cells including recovery buffer and assay medium
- 5 vials of 1E7 cells (CatN° RE509-01)

[1] licensed by the German Cancer Research Center (DKFZ) for assay ready use.