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COMPETENCE IN SKIN PHYSIOLOGY
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Protocol

Micronucleus Assay

with

epiCS®

(version 1.3)

Content

1. Background

In general, the term genotoxicity refers to the ability to induce structural changes in genes via interaction with DNA and/or non-DNA targets (1). The hazard classification "is primarily concerned with chemicals that may cause mutations in the germ cells of humans that can be transmitted to the progeny"(2). The classification system "is hazard based, classifying substances on the basis of their intrinsic ability to induce mutations in germ cells" and "is not meant for the quantitative risk assessment of substances" (2).
So far, eight *in vitro* methods for genotoxicity testing have been adopted at the EU level. The classical *in vitro* genotoxicity tests, like the gene mutation test in mammalian cells (OECD 476), the chromosome aberration test (OECD 473) and the *in vitro* micronucleus test (OECD 487) are commonly used to predict the intrinsic potential of substances to induce mutations. Mutations and chromosomal aberrations are strongly associated with carcinogenic processes and only *in vitro* genotoxicity tests which measure a mutation endpoint are qualified for identifying potential carcinogens. So far, none of the *in vitro* genotoxicity tests are formally validated with the exception of the *in vitro* micronucleus test, although for all of these tests OECD guidelines exist. Nevertheless, *in vitro* genotoxicity tests are scientifically accepted and widely used (3).

The micronucleus is formed during metaphase/anaphase transition of mitosis and represents broken fragments of daughter chromosomes outside the nucleus. The micronucleus formation can be used as a diagnostic tool for chromosomal DNA damage and is monitored via staining of DNA, cell membrane and nuclear membranes followed by microscopic analysis.

2. Basic Procedure

Upon receipt the tissues should be conditioned by pre-incubation in epiCS Culture Medium for release of transport stress related compounds and debris. After at least 2 h or overnight pre-incubation tissues are transferred to fresh epiCS Culture Medium and topically exposed for 2 days with the test chemicals. At least two (we recommend three) tissues are used per treatment, negative control and positive control. To block cytokinesis during the exposure, cytochalasin B is added to the culture medium which enables the formation of binucleated interphase cells. After exposure to the substances the cells are harvested as a single cell suspension. Staining, fixation and microscopic analysis follow.

3. Materials

3.1. epiCS kit components

Examine all components for integrity. If there is a concern please contact CellSystems (Telephone +49-2241-25515-0, Fax: +49-2241-25515-30, E-Mail: info@cellsystems.de)

Sealed 24-well plate (contains epiCS on agarose)
epiCS Culture Medium
Manual + Certificate of Analysis

3.2. Additionally needed materials and laboratory equipment

Material / Equipment	**Application**
Sterile, blunt-edge forceps	*For transferring tissues from agarose*
Sterile, blunt-end forceps	*For separating the epidermis from the insert membrane*
Sterile disposable pipettes, pipette tips and pipettors	*For diluting, adding, and removing media and test material. For topically applying test substances to tissues.*
Adjustable pipettes (10 µl, 1 ml, 2 ml, 5 ml)	*For diluting, adding, and removing media and test material. For topically applying test substances to tissues.*
37°C incubator, 5% CO_2, 95% RH	*For incubating tissues prior to and during assays*
Laminar flow hood	*For transferring tissues under sterile conditions, for sterile application of test substances to tissues*
Fluorescence Microscope	*For microscopic analyses (use 630x magnification or higher)*
24-well plates	*For immerse incubation of epiCS with PBS, EDTA and Trypsin-EDTA solutions*
6-well plates	*For incubation of epiCS and cell harvesting*
Cytochalasin B from *Drechslera dematioidea* (Sigma-Aldrich #C2743-300UL)	*Enables formation of micronuclei in binucleated interphase cells* *As stock solution use 3 mg/ml (diluted in DMSO)* *Dilute stock solution 1:1000 in culture medium*
Acridine Orange hydrochloride hydrate (Sigma-Aldrich #318337)	*For fluorescent staining of cells* *Working solution: 0.04 mg/ml (diluted in PBS)*
Trypsin (0.05% Trypsin/0.02 %EDTA) (Lifeline #CM-0017)	*For dissociation of tissues.*
Trypsin Neutralizing Solution (TNS) (Lifeline #CM-0018)	*Inactivation of Trypsin/EDTA.*

Material / Equipment	**Application**
EDTA Solution (Versene Solution) (Life Technologies #15040-033)	*For gentle non-enzymatic cell dissociation before Trypsin-EDTA treatment.* *Use as ready solution* *- or prepare working solution (0.48 mM): 0.2 g EDTA per liter PBS*
Trypan Blue Solution (Sigma-Aldrich #T8154)	*Vital staining of cells*
Potassium chloride (KCl)	*For soaking cells* *Working solution: 37.5 mM (diluted in aqua bidest)*
Fixing solution (methanol-acetic acid, 3:1)	*Fixation of cells*
Roti ®-Histokit (Carl Roth #6640)	*For embedding cells*
Phosphate Buffered Saline (PBS) without calcium or magnesium (Lifeline #CM-0001)	*Use for washing of tissues, dilution of solutions and dissolving acridine orange* *Use as ready solution* *- or dilute from 10x concentrate* *- or prepare from PBS powder*
Aqua bidest	*For dissolving and dilution of chemicals/solutions*

4. Method

4.1. Tissue and Medium Storage

Set up the 6-well plates and pipette 1 ml cold epiCS Culture Medium (2-8°C) into each well. Remove the Parafilm from the 24-well transport plate containing the epiCS and open under sterile conditions. Lift the inserts with sterile forceps and transfer them into the sterile 6-well plate filled with epiCS Culture Medium. Make sure not to transfer any agarose. Avoid air bubbles between inserts and the bottom of the culture dish by setting the inserts at an angle into each well. Incubate the epidermis equivalents for at least 2 hours at 37°C, 5% CO_2, and 95% humidity before performing first experiments.
Prolonged storage (on agarose) is not recommended. Store epiCS Culture Medium in the dark at 2-8°C. The shelf life is limited (see expiry date on the label).

4.2. Micronucleus Assay

Preparations
Prepare all solutions and media freshly before use (see section Materials).
Clean glass slides for 20 min with 70% ethanol and rinse the slides twice with aqua bidest. Dry slides for 3 h at 80°C and store them until use at 2-8°C.

Culture start

At the day of receipt epiCS are conditioned with fresh epiCS Culture Medium by pre-incubation for at least 2-3 h or overnight in the incubator (37±1°C, 5±1% CO_2, 95% RH).

First application

The culture medium beneath the epiCS is exchanged by supplemented Culture Medium with Cytochalasin B (3 µg/ml). 10-50 µl of the test substance is applied topically onto the epiCS followed by 24 h incubation (37±1°C, 5±1% CO_2, 95% RH).

Second application

Exchange the Culture Medium (including fresh Cytochalasin B) and repeat topical application of the test substance. Thereafter tissues are incubated (37±1°C, 5±1% CO_2, 95% RH) for another 24 h.

Cell harvesting, fixation and staining

Take a tissue from the treatment plate and blot the bottom on a paper towel to remove excess medium. Place the tissue into a 24-well plate containing 2 ml PBS (w/o $CaCl_2$ and $MgCl_2$)/well and completely immerse the tissue for 30 min at 37°C.

Take the tissue and decant PBS from inside of the insert by inverting and blot (on a paper towel) to remove excess PBS. Place the tissue in a new 24-well plate and pipette 2 ml EDTA solution (0.02 %) into the well to immerse the tissue. Incubate the epiCS for 30 min at 37°C.

Decant EDTA solution and blot the tissue on a paper towel. Place the tissue in a new well containing 2 ml pre-warmed (37°C) Trypsin-EDTA solution. Incubate for 30 min at 37°C.

Decant half of the Trypsin-EDTA solution out of the insert. Take the epiCS, hold the insert directly above a well of a new 6-well plate containing 1 ml pre-warmed (37°C) Trypsin-EDTA solution and carefully separate the epidermis from the insert membrane by gently grabbing the edge of the tissue with forceps and peeling away from the edge. Do not remove the remaining Trypsin-EDTA inside the insert and place the separated epidermis into the well. Incubate epidermis in well for 2 min with agitation (37°C). Add 2 ml TNS (Trypsin Neutralizing Solution) into the 6-well containing the epidermis and the insert. Resuspend the cells by pipetting up and down the tissue / cell suspension with am Eppendorf Micropipette (1.25 ml or 1 ml) which tip has been cut off at about 0.5 cm from the opening.

Transfer cells in a 15 ml centrifuge tube.

Rinse insert membrane and well with 1 ml PBS, to collect also cells, that are still attached to the membrane or the well surface and add this cell suspension into the centrifuge tube, too.

Centrifuge the cells at max 300 x g for 5 min.

Decant the supernatant and resuspend cells in 1 ml PBS.

Cell counting:

Transfer the cell suspension into a 1.5 ml tube and dilute 50 µl of the suspension with 50 µl trypan blue and centrifuge the residual cells at max 300 x g for 5 min.

During centrifugation count the cells using a hemacytometer to record cell count and cell viability.

Decant the supernatant and resuspend cells in 1 ml KCl (37.5mM). Incubate for 1 min at RT.

Add 3 ml freezing cold (-20°C) fixation solution drop by drop and resuspend carefully.

Centrifuge the cells at 300 x g for 5 min.

Resuspend the pellet in 200-500 µl cold fixing solution.

Prepare a heating plate (40°C) under a fume hood during incubation at -20°C.

Place a clean glass slide on the heating plate for 3 - 5 min and pipette the complete volume of the cell suspension (drop by drop) on the slide [notice: hold the pipette 2-5 cm above the slide and shortly wait after each drop for evaporation of the fixing solution].

Incubate the slides for 20 min on the heating plate and chill slides to RT afterwards, or shortly flame the slide and allow drying on a heating plate.

Incubate slides 3-5 min in a staining chamber containing acridine orange solution (0.1 µg/ml) at RT in the dark.

Rinse slides twice with aqua bidest.

Dry slides and embed cells with mounting medium and a cover slip for microscopic analysis.

Store slides at 2-8°C in the dark.

Microscopic analysis and result assessment

For evaluation and analysis of the test results, at least 1000 binucleated cells per duplicate must be scored. Cells are scored and classified regarding to the number of nuclei (mono- and binuclei) and binucleated cells containing micronuclei. Trinucleated or multinucleated cells are not scored. Binucleated cells are only scored if the nuclei are separated and of approximately equal size, even if they overlap as long as nuclear boundaries can be distinguished (Figure 1).

If a test substance reproducibly increases the number of cells containing micronuclei compared to negative controls it is classified as a genotoxic substance.

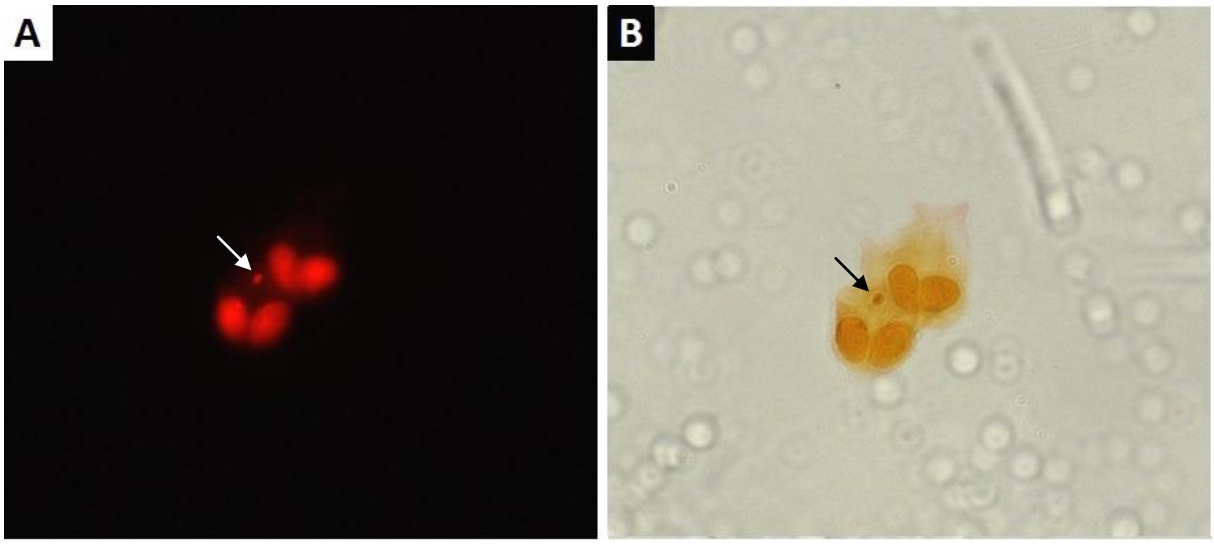

Figure 1: Binucleated cells containing a micronucleus. (A) Fluorescence microscope image of two binucleated cells. One cell contains a micronucleus (white arrow)**. (B)** Bright field microscope image from A. Microscopic analysis were performed with an Axiovert 200 (Zeiss) at 630x magnification.

5. References

1. Maurici D., Aardema M., Corvi R., et al. (2005). Genotoxicity and mutagenicity. *Altern La Anim* 33, Suppl 1, 117-130.
2. United Nations Economic Commission for Europe (UNECE). (2013). Globally Harmonized System of classification and labeling of chemicals (GHS). Fifth revised edition. Part 3. Health hazards. Chapter 3.5. Germ cell mutagenicity.
3. Adler S., Basketter D., Creton S., et al. (2011). Alternative (non-animals) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol* 85, 372-379.