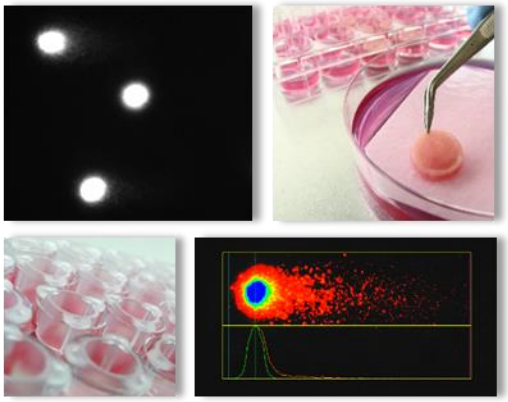
E-Mail: info@phenion.com

Web: www.phenion.com

phenion
COMPETENCE IN SKIN PHYSIOLOGY
www.phenion.com A Henkel brand

3D Skin Comet assay protocol using the Phenion® Full-Thickness Skin Model



Preface

For the assessment of mutagenic and clastogenic properties of chemicals, the 3D Skin Comet assay using the Phenion® Full-Thickness Skin Model successfully underwent a validation exercise in which Henkel and additional four European and US-American laboratories cooperated (Hoffmann, *et al.*, 2017). In parallel to the validation, the intended regulatory use of the assay has already been exemplified, i.e. three dossiers on hair dye ingredients which were supported by 3D Skin Comet assay data were accepted by the respective European regulator and the hair dyes considered as 'safe of use' with regard to genotoxicity (Reisinger *et al.*, 2016).

The following 3D Skin Comet assay protocol reflects the Standard Operating Procedure developed in a joint research project which was funded by Cosmetics Europe and the German Federal Ministry for Research & Education (Reisinger *et al.*, 2018). The same applies to an evaluation sheet in which genotoxicity and cytotoxicity raw data can be collated and subsequently the results be calculated in excel.

E-Mail: info@phenion.com

Web: www.phenion.com

phenion
COMPETENCE IN SKIN PHYSIOLOGY
www.phenion.com A Henkel brand

Content

1. Introduction and principle

The comet assay was first introduced by Ostling and Johanson (1984) as single cell gel electrophoresis assay to detect DNA double-strand breaks after cells (nuclei) embedded in a micro-gel were subjected to electrophoresis. After staining, intact DNA, which was unable to migrate under the electrophoresis conditions used, appeared as round shaped cell nuclei. In contrast, DNA fragments - evolved from DNA damage - could migrate towards the anode in the electric field. They became visible as the tail of a comet beside the comet head build of non-migrated DNA.
Subsequently, the introduction of highly alkaline conditions (pH >13) during electrophoresis and a preceding DNA unwinding step by Singh *et al*. (1988) allowed the detection of a broader range of DNA damage. This included single-strand breaks which may result from direct interaction of the test chemical with DNA or are related to incomplete excision repair as well as alkali labile sites (OECD TG 489). Thus, not only clastogenic DNA damage, but also lesions which may give rise to gene mutation could be detected.

As the comet assay methodology does not rely on proliferating cells, any tissue allowing for isolation of single cells can be analyzed with the methodology. This assigns the comet assay a worthwhile approach for investigating the first site-of-contact in toxicological safety assessment. Its increased acceptance in regulatory testing has been documented in 2014 by the release of the 'In Vivo Mammalian Alkaline Comet Assay' OECD Testing Guideline (OECD TG 489, updated in 2016).

The alkaline comet assay was combined with 3D skin tissues to address two aspects underrepresented in classical *in vitro* genotoxicity assays, i.e., a species- and organ-specific xenobiotic metabolism and the relevant route of exposure.
In general, full thickness skin tissues have been shown suitable for the 3D Skin Comet assay. In specific, the Phenion® Full-Thickness (FT) Skin Model was used in the validation exercise in which 30 coded chemicals have been tested blinded in five laboratories (Hoffmann *et al.*, 2017; Reisinger *et al.*, 2018). The tissues are made of primary and p53 competent cells of human origin, thus eliminating the species barrier and anticipating normal cell cycle control. Its three-dimensional environment supports a phenotype close to native human skin, which was demonstrated by comparable gene and protein expression patterns (Mewes *et al.*, 2007). As this similarity was also shown for gene expression and protein activity of enzymes belonging to xenobiotic metabolism (Wiegand *et al.*, 2014; Jäckh *et al.*,

2011; Bätz *et al*., 2013), the utilization of external metabolizing systems such as rat liver S9 mix is not required.
Furthermore, the topical application of test chemicals onto full thickness skin tissues, which are cultured under air-liquid-interface (ALI) conditions, is supposed to overcome solubility issues observed with classical submerged cell cultures.

As a conclusion Phenion® Full-Thickness skin models excellently mimic the route of exposure for dermally applied chemicals, and therefore allows for testing conditions very close to the intended condition of use.

Already in parallel to the validation exercise the intended regulatory use of the 3D Skin Comet assay was exemplified, i.e., to follow up on positive results from initial standard testing. In fact, 3D Skin Comet assay data using the Phenion® FT were introduced into the toxicological safety assessment of a hair dye ingredient to follow up on positive findings of the Bacterial Reverse Mutation Test (OECD TG 471, 1997) and submitted to the Scientific Committee on Consumer Safety (SCCS), an independent expert panel of the European Commission. The SCCS accepted the negative data of the 3D Skin Comet assay in a weight of evidence approach and declared the hair dye ingredient as ‘safe of use’ in terms of genotoxicity (SCCS, 2014a). In the meantime, two additional hair dyes were successfully processed the same way (SCCS, 2015, 2016; Reisinger *et al*., 2016).
Moreover, the SCCS provides guidance on the testing of cosmetic ingredients and revised its guidance for genotoxicity testing to reflect progress made with the characterization and validation of the reconstructed skin model-based assays (SCCS, 2014b). The SCCS calls assays based on human reconstructed skin tissues a ’good alternative to bridge the gap between *in vitro* and *in vivo* testing in terms of final hazard assessment‘ (SCCS, 2014b) and recommends these assays as follow-up on suspected irrelevant positive results from the *in vitro* standard test battery.

2. Area of application

The 3D Skin Comet assay using Phenion® FT Skin Models enables the detection of genotoxic effects after dermal exposure of compounds.

In general, Phenion® FT Skin Models support testing of a great variety of compounds and compensate certain downsides observed with submerged 2D monolayer cultures. The 3D skin tissues ease testing of e.g. lipophilic compounds and the application of doses relevant

for the intended use, which might be higher compared to non-cytotoxic doses tolerated by monolayer cultures. During the validation exercise > 30 coded chemicals were tested, e.g. direct mutagens or pro-mutagens of different chemical classes and modes of action (Hoffmann *et al.*, 2017). Coloured substances were tested in parallel to the validation exercise and did neither interfere with the tissue´s integrity in the cultivation phase nor with DNA damage evaluation (SCCS, 2014a; 2015; 2016).
Solvents like acetone or 70% ethanol (v/v) were successfully used during the validation study of the 3D Skin Comet assay since they easily evaporate and do therefore not disturb the air-liquid-interface, which is essential for a proper tissue cultivation over a period of 48 h (Reisinger *et al.*, 2018).

3. Materials

Safety note: For working with test compounds, compliance with all relevant regulations and instructions for handling of hazardous chemicals must be ensured. The disposal of substance-contaminated lab materials must comply with respective safety regulations.

3.1. Chemicals

Important note: Reagents needing a specific characteristic (e.g. purity) are highlighted in **bold**. The use of these products is recommended.
The order numbers for the other reagents are examples and other suppliers could be used.

Chemical/kit	Company	Order-No.
Acetone	Merck	1.00014.2511
Agarose Sea Plaque GTG LMP	**Biozym** **Lonza**	**850111** **50100**
Aphidicolin	**Sigma-Aldrich**	**A0781**
ATPlite Luminescence Assay System Kit	**Perkin Elmer**	**6016943**
Benzo[a]pyrene (BaP)	Sigma-Aldrich	B1760
Bradford Protein Reagent	**Sigma-Aldrich**	**B6916**
Bovine Serum Albumin solution (2 mg/mL standard for Bradford assay)	Thermo Fisher Scientific	23209
$CaCl_2$	Roth	CN93.1

Cell strainer (70 µm mesh size)	Corning	352350
DMSO (≥99.7% purity (e.g. Hybri-Max™), recommended as supplement of mincing buffer)	**Sigma-Aldrich**	**D2650**
DMSO ≥99.8% recommended as supplement of the lysis buffer	Roth	4720.1
Ethanol p.A.	Merck	1.00983.2511
EDTA	Sigma-Aldrich	E5134
HBSS w/o Ca/Mg	Thermo Fisher Scientific	14175053
HCl	VWR	20252.295
Hepes	Invitrogen	15630
KCl	Roth	6781.1
MEEO-Agarose (Ultra-Quality)	**Roth**	**2268**
Methyl methanesulfonate (MMS)	Sigma-Aldrich	129925
NaCl	Roth	9265.2
NaOH	Merck	1.06482.1000
PBS without Ca/Mg	Thermo Fisher Scientific	14190094
SYBR Gold	**Thermo Fisher Scientific**	**S11494**
Thermolysin	**Sigma-Aldrich**	**T7902-100 mg**
ToxiLight™ Non-destructive Cytotoxicity BioAssay Kit (adenylate kinase measurement)	**Lonza**	**LT07-217**
Tris	Sigma-Aldrich Roth	T6066 4855.2
Triton-x 100	Sigma-Aldrich	T8787

1000x APC stock solution (5mg/mL)	
1 mg	Aphidicolin
0.2 mL	DMSO

Dissolve APC in DMSO, aliquot and store at -20 °C.

3.2. Buffers

Buffers for cell isolation and comet assay procedure are prepared according to Tice *et al.*, 2000.

Agarose for coating slides	
1 g	MEEO-Agarose
Add PBS without Ca/Mg to a final volume of 100 mL.	

Preparation of agarose-coated slides
Boil agarose e.g. on a heating plate. Dip slides in melted agarose 1-2 times to obtain a homogeneous layer on the top. Wipe off agarose on the bottom side of the slides using a paper towel. Let agarose layer solidify at room temperature. Coated slides can be stored in a dust-free and humid atmosphere.

Thermolysin Buffer	
0.5 g	KCl
0.58 g	NaCl
0.147 g	$CaCl_2$
2 mL	1 M Hepes
Add DI water to a final volume of 200 mL.	

Dissolve 0.1 g thermolysin (one entire vial) in the buffer to a concentration of 0.5 mg/mL, aliquot, and store at -20 °C for up to 12 months.

Mincing Buffer	
3.72 g	EDTA

Add HBSS w/o Ca/Mg, adjust to pH 7.0-7.5 (important!) and fill up to a final volume of 450 mL. Add fresh, i.e., briefly before use, DMSO to a final concentration of 10%.

Low Melting Agarose (LMA) to resuspend cells	
0.5 g	LMA
Add PBS without Ca/Mg to a final volume of 100 mL.	

Lysis Buffer pH 10 (Stock solution)	
292.2 g	NaCl
74.4 g	EDTA
2.4 g	Tris
12.0 g	NaOH
Add DI water to a final volume of 2000 mL.	

Lysis Buffer (Working solution)	
66.75 mL	Lysis-Buffer (Stock solution)
7.5 mL	DMSO
750 µL	Triton-x 100

10 M NaOH	
400 g	NaOH
Add DI water to a final volume of 1000 mL.	

200 mM EDTA pH 10	
74.4 g	EDTA
Add PBS w/o Ca/Mg, adjust to pH 10 and fill up to a final volume of 1000 mL.	

Electrophoresis Buffer	
60 mL	10 M NaOH
10 mL	200 mM EDTA
1930 mL	DI water

Neutralisation Buffer pH 7.5	
97.0 g	Tris
58 mL (ca.)	HCl
Add DI water to a final volume of 2000 mL.	

TE-Buffer	
1.21 g	Tris
0.372 g	EDTA
Dissolve Tris and EDTA in DI water, set pH to 7.0-7.5 and adjust final volume to 1000 mL.	

Staining solution (1:10.000, SYBR Gold)
Dilute sufficient SYBR Gold with TE-buffer, pH 7.2 for immersing slides in suitable class cuvette. Adapt the volume according to the size of the cuvette used. Protect the solution from light. It can be used up to 3 times (maximum 30 slides) within one day.

4. Study structure

The assessment of the genotoxic potential of a test compound with the 3D Skin Comet assay comprises the following steps:

- Solubility study (section 4.1)
- Dose range finding experiment (section 4.2)
- Main experiments with or without APC (section 4.3).

4.1. Solubility study

The aim of this first step is to identify the maximal concentration in terms of solubility (Note: The maximal concentration in terms of cytotoxicity will be identified in the dose range finding experiment and further verified in the main experiments).
The maximum test concentration for well-soluble compounds is predefined with 10% (w/v), i.e. 100 mg/mL or 1600 µg/cm^2 surface of the skin model. If the chemical is not soluble at 10% (w/v), additional aliquots of the solvent should be added in small steps to define the highest soluble concentration. The procedure is recorded in detail to calculate precisely the maximal concentration in terms of solubility.

Acetone is used as the standard solvent. If the solubility of the test compound is below 1% in acetone while its solubility is improved in 70% ethanol (v/v), the latter solvent should be used for further experimentation. In general, it must be secured, that solvents do not induce cytotoxicity and do not modify the characteristics of test compounds.

Precipitations on the surface of tissues shall be avoided as they potentially risk false-positive results. Tissues are investigated for precipitation at the end of experiments. The occurrence of droplets that do not disappear rapidly is considered a precipitation as well. A precipitating dose group will provide the cut-off concentration for the following experiment. It is recommended to use the lowest precipitating concentration as the highest dose tested.

4.2. Dose Range Finding

The dose range finding experiment (DRF) should cover a wide concentration range. Thus, additional dose groups are added up to the maximum soluble concentration defined in the solubility study. Although the investigation of cytotoxicity is decisive for the dose range finder, also the analysis of DNA strand breakage by performing a comet assay can be helpful for decision making. The DRF does not necessarily have to fulfill the validity criteria described in section 6. For example, the use of 1-2 tissues per test group will be sufficient.

4.3. Main Experiment

The maximum dose for the main experiment is determined by the highest feasible dose obtained from the DRF. This dose is determined by (1) the maximum soluble concentration and (2) the cytotoxicity of test chemicals shown in the DRF. Thresholds set to indicate strong cytotoxicity and thereby invalid dose groups are defined in section 6.2.2. In general, an equal spacing between doses shall be used within an experiment. While only three valid concentrations are required, it is recommended to test at least four concentrations to compensate potential loss of a dose group, e.g. due to high cytotoxicity.

4.3.1. Experimental design

An experiment is designed as followed:

- Solvent control (SC) and a positive control (PC) are incorporated in each experiment.
- At least three concentrations of the test compound are analyzed.
- Three tissues are used for each control/dose group.

- Identical treatment of controls and test groups (e.g. incubation time and way of application) is essential.

The skin tissue treatment time is 48 h in total, with three dosing applications (see Fig. 1). Treatments at the start of the experiment and after 24 h allow induction of phase I of the xenobiotic metabolism (Wiegand *et al*., 2014). The last treatment, 3 h before the end of the experiment, takes DNA damage into account that might be subjected to immediate repair. In contrast, MMS as positive control is applied only 3 h before the end of experiments. This exposure time has been validated to induce reliably measurable DNA damage.
In case of initial negative or equivocal results, aphidicolin (APC) is added in a follow up experiment, 4 h before sampling. APC is described as an inhibitor of DNA polymerases α and δ (Speit *et al.*, 2004), which both also possess excision repair function. Consequently, APC induces accumulation of DNA strand breaks evolved during DNA repair processes to amplify comet formation as proposed by Brinkmann *et al.* (2013) (see also Reisinger *et al.*, submitted). This follow up experiment should use Benzo[*a*]pyrene (BaP) as positive control. BaP, a pro-mutagen, is introduced to proof the effectiveness of APC. The following experimental design applies in these cases: SC/APC, BaP, BaP/APC, conc 1/APC, conc 2/APC, conc 3/APC.

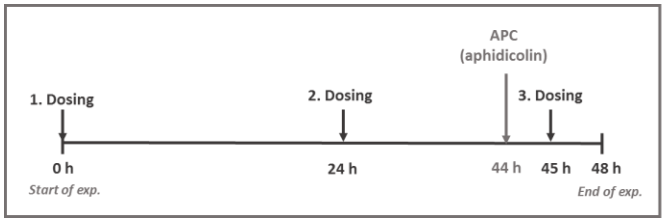


Figure 1. Treatment schedule. Tissues are exposed with the test compound for 48 h in total. A maximum of 100 mg/mL of the test compound in acetone (70% ethanol (v/v)) is applied three times. In case of negative or equivocal findings, aphidicolin is added 4 h before the end of experiment atop of tissues.

4.3.1.1. Negative control

It is recommended to make use of a negative control of three untreated tissues to be included in each experiment to generate a historical control of approximately 15 experiments. When the respective laboratory showed that the recommended solvents used (acetone, 70% ethanol, aqua DI) has no relevant impact on cytotoxicity or genotoxicity in comparison to the negative control (Reisinger *et al.*, 2018), the negative control can be

omitted. If this is reliably demonstrated, is can be assumed, that the data for the solvent control will correctly reflect background DNA damage of tissues in the respective experiments.
In case a new solvent is used, the incorporation of a negative control is recommended to ensure concordance with the historical database.

4.3.1.2. Solvent control

Two solvents have been approved for the assay: acetone and 70% ethanol/water (Reus *et al.*, 2013; data not shown). The solvent selected for the test compound must be used as SC. For the positive control, acetone is always used as solvent (independent from the solvent used for the test compound). If acetone is only used for the PC, it does not have to be included as a second SC in the experiment.

4.3.1.3. Positive control

Methyl methanesulfonate (MMS, 5 µg/cm^2) a direct acting alkylating genotoxicant is broadly accepted as positive control in genotoxicity studies. It is also used with the 3D Skin Comet assay and applied for 3 h.

In case APC is introduced in an experiment, benzo(*a*)pyrene (BaP, 12.5 µg/cm^2, three applications at 0, 24, and 45 h) is used as a positive control instead of MMS.

4.4. Study design

Different scenarios can be expected when investigating the genotoxic potential of chemicals. Clear positive findings in a first valid experiment need only to be confirmed in a second test run in case of transferability or validation studies (scenario **A**, see section 6 for definition of the term ‘valid’).

In case the first main experiment provided a negative result, aphidicolin (APC) is added (see also section 4.3.1 for details on APC). In case of a clear positive or negative result the study is finalized (scenarios **B** and **C**).

In case the APC experiment is considered equivocal (scenario **D**) or the first two main experiments without APC are non-concordant (scenario **E**), a third experiment with a modified concentration range and/or APC treatment is indicated.

Scenario	1st experiment	2nd experiment	3rd experiment	Final call
A	positive	(optional)	-	positive
B	negative	negative with APC	-	negative
C	negative	positive with APC	-	positive
D	negative	equivocal with APC	APC experiment with modified dose range	tbd
E	positive	negative	3rd experiment with maybe modified dose range and/or APC	tbd

5. Comet assay protocol

5.1. General precautions

Phenion® FT Skin Model are protected from direct UV light during a comet assay study to avoid secondary DNA damages.

5.2. Handling of Phenion® FT Skin Models after shipment and during culture

On the day of arrival, Phenion® FT Skin Models are controlled for proper shipment (i.e. damaged containers, the filter paper under tissues should be wet; liquid on top of a tissue must be removed with a sterile collection swab and shall not reappear during cultivation).

Afterwards, i.e., on the same day of arrival, tissues are transferred into standard cell culture conditions:

- Use only pre-warmed ready-to-use ALI medium w/o Phenol red provided with the skin models.
- For comet assay experiments tissues are cultivated separately in small Petri dishes (3.5 cm diameter) which are placed in one large Petri dish (10 cm diameter). See also *Instruction for use of Phenion® FT Skin Models* and respective video tutorial on https://www.phenion.com/information-center.

- Place three small Petri dishes in one large Petri dish and insert one filter spacer into each small Petri dish ensuring correct positioning of filter spacer with pin orientation upwards.
- Add approx. 5 mL medium into each small Petri dish. The medium level should reach the upper tip of the filter spacer pins.
- Carefully place small sterile filter papers on top of the spacer units to enable wetting of the filter with ALI-Medium.
- Grab the Phenion® FT Skin Models carefully using sterile forceps and transfer them on top of the filter papers. Place one skin model per small Petri dish. Make sure that no air-bubbles appear between the tissue and the filter paper.
- After transferring the tissues, check that the filter papers are completely soaked with ALI-Medium. If needed, do not hesitate to add further medium to ensure that the basis of the Phenion® FT Skin Models on the filter paper is slightly surrounded by the liquid.
- Cover the tissue culture system with the lid of the large Petri dish and discard the lids of the small ones.

Tissues are preferably equilibrated overnight by cultivating at 37 °C, 5 % CO_2, 95 % humidity prior to experiments.

- Exchange medium on the next day.
- No further medium change is needed for a 48 h experiment.
- Start treatment of tissues (see next chapter).

5.3. Treatment of tissues

Phenion® FT Skin Models have a surface area of 1.56 cm^2 and are exposed to 25 µL of respective solutions (i.e., 16 µL/cm^2). Please consider the following points while treating the tissues:

- Prepare dose solutions of the test chemical always freshly.
- Solvents shall be used cold (approx. 4 °C) and all appropriate test solutions should be kept on ice to reduce potential evaporation.
- Test compound application must be performed carefully, i.e., avoid or minimize direct contact of pipette tip with the tissue. A homogeneous distribution of the

solution/solvent over the whole tissue surface is important, i.e., solvents like acetone either spread by its own or by gently moving the long side of a pipette tip across the tissue surface.

In case APC is added to experiments, please perform the following steps 4 h before sampling:

- Add 1 µL '1000x APC stock solution' per mL of ALI-medium to a final concentration of 5 µg APC/mL ALI-medium.
- Mix medium with caution by pipetting up and down (avoid generation of air bubbles) to allow even distribution of APC.
- Continue incubation of tissues for 4 h before sampling.

Any observations in conjunction with chemical treatment must be recorded properly, e.g. the occurrence of precipitations (see also section 4.1), as they may have an influence on the experimental outcome.

5.4. Single Cell Isolation

Please handle a maximum of eight compartments (epidermis plus dermis) derived from four Phenion® FT Skin Models in parallel.

- Wash the skin model with PBS without Ca/Mg, e.g. transfer 5 mL PBS in a cavity of a 12-well plate and submerge tissue in PBS.
- While holding the skin model with bent forceps, carve out a quarter of the tissue by using a clean scalpel or scissors.
- Transfer the quarter of the skin model into a labeled reaction tube, snap freeze in liquid nitrogen, and store at -80 °C for later determination of ATP and protein concentration (see section 5.8.1 – 5.8.3).
- Store a 500 µL aliquot of the ALI-medium of each tissue at 4 °C for up to 5 days (no longer) for adenylate kinase activity measurement using the ToxiLight cytotoxicity assay (see section 5.8.4).
- Pipette 300 µl thermolysin (0.5 mg/mL) in the cavity of a 12-well plate and distribute evenly. Transfer tissue carefully 'on top' of the enzyme solution to guarantee that no thermolysin gets on top of the tissue. Incubate for 2 h at 4°C.

- Separate both compartments with the help of forceps. (Fixate the dermis of the tissue with forceps and carefully peel off epidermis from dermis with another bent forceps.)
- Transfer the epidermis and dermis separately in two labelled 2 mL reaction tubes containing 1 mL ice-cold mincing buffer.
- Cut epidermis or dermis in fragments using clean scissors (cut 15-20 times) and incubate fragments on ice for 5 min.
- To generate a single cell suspension, resuspend fragments in the mincing buffer by carefully pipetting several times up and down.
- Apply the cell suspensions to a cell strainer with a mesh size of 70 µm to remove cell debris (a 40 µm mesh size is only recommended in the case high amounts of tissue debris appear on the slides). Collect the cells by centrifugation at 200 x g, 3 min, 4 °C and proceed with section 5.5.

5.5. Slide preparation and lysis

- Decant supernatant carefully, except for a small volume (25-50 µL) and resuspend the cell pellet by gently tapping at the tube wall.
- Add 250-350 µL (depending on the cell yield) of 0.5% LMA (37 °C), and resuspend cells carefully by pipetting up and down up two times (avoid extended shear stress).
- Spread one aliquot of 75 µL LMA/cell suspension on each of three agarose coated slides. Cover slides with a cover slip and let cool to solidify the LMA (e.g. place slides either onto a cool pack, in a box filled with ice or in the refrigerator for approx. 5 min).
- Carefully remove the cover slips and incubate the slides directly at 2-10 °C in freshly prepared lysis buffer overnight (max. 72 h).

5.6. DNA unwinding and electrophoresis

In order to support standardization of results, it is recommended to use a big electrophoresis chamber e.g. Carl Roth GmbH & Co. KG, Karlsruhe, Germany (Cat. No. N610.1), which was used during the validation exercise and with which 40 slides can processed in parallel. It is recommended to conduct the following steps in a cold room at 4 °C. Alternatively (if no cold room is available), place the precooled electrophoresis chamber on ice.

- Check and, if necessary, adjust precisely the horizontal position of the electrophoresis chamber with a mechanic's level. The exact positioning is crucial, as the liquid pillar above the slides is a decisive parameter for comet assay results.
- Fill the chamber with approximately 2.4 L of fresh 4 °C electrophoresis buffer (do not re-use electrophoresis buffer).
- Remove slides from the lysis buffer, briefly wash once with precooled electrophoresis buffer (to remove residuals of the lysis buffer) and transfer slides in the electrophoresis chamber containing fresh electrophoresis buffer in random order. Add empty slides if needed to run each electrophoresis with the same number of slides. Incubate the slides for exact 20 min (!) to allow unwinding of DNA.
    - ! The three slides of each tissue compartment shall be subjected to at least two different electrophoresis runs (if feasible a separate run for each replicate slide is preferred).
- After 20 min incubation adjust power supply to 39 V and 450 ± 10 mA and start the electrophoresis.
- Adjust to 450 mA by adding buffer through the pre-defined holes in the lid of the electrophoresis chamber and perform the electrophoresis for 30 min.
- Switch-off power supply to stop electrophoresis. Remove slides and place them into a glass chamber containing neutralization buffer for at least 5 min (longer incubation up to 15 min is possible to ease handling of further steps).
- Transfer slides to 96 % (v/v) ethanol for 5 min.
- Remove slides and let them air dry at room temperature. Cover slides to protect them from dust and from direct light.

5.7. Staining and comet analysis

Slides are first randomized (e.g. www.random.org) and coded by a person, not involved in the analysis of slides to exclude operator bias. Two slides per compartment, i.e. epidermis and dermis, are used for evaluation; the third slide is stored as back-up.

Comet slides are analyzed under fluorescent microscopes allowing excitation maxima of ~495 nm and ~300 nm (the emission maximum of SYBR Gold is given with ~537 nm) using 100x (used to scan the overall appearance of a slide) and 200x (used for analysis) magnification respectively.

Make sure that the microscope lamp does not exceed its maximum operational time (refer to the manufacturers manual) and the camera settings are correct, i.e. always use identical settings for comet analysis. For the assessment of comet assay signals semi-automated image analysis software is highly recommended, e.g. Comet Assay IV (Perceptive Instrument, UK). Respective standards were published by Reus *et al.*, 2013.

- Prepare fresh comet staining solution as detailed in section 3.2.
- Immerse the slides in the staining solution for 15 min at RT.
- Remove slides and place cover slips on the slides to prevent drying. ! Do not stain more slides at a time than you can analyze within up to 2 hours.
- Place the slide under the microscope. Adjust the sharpness and analyze 50 randomly selected comets regarding standards as given by Reus *et al.*, 2013.
- Remove the cover slips immediately after analysis and let slides air dry. Slides can be stored protected from dust and light.

5.8. Cytotoxicity determination

5.8.1. Homogenization of the tissue samples (stored at -80°C from step 5.1)

Requirements

- Tissue Lyser (e.g. Qiagen, version II)
- Stainless steel beads (d= 5 mm)
- Heating block
- PBS w/o Ca/Mg
- 1.5 mL tubes

Chill buffers and relevant equipment (e.g. TissueLyser adaptors, tubes, beads, etc.) on ice before and perform all steps on ice.

- Add 1000 µL PBS w/o Ca/Mg to the frozen tissue piece of the Phenion® FT Skin Model, stored in a 2 mL Tube as described in section 5.1.
- Add one stainless steel bead to each tube and homogenize for 5 min, 30 Hz using the TissueLyzer II.
- Heat homogenate for 5 min at 105 °C in a heating block, to stop any ATP consuming enzymatic reactions.
- Transfer tube immediately on ice to chill for 5 min.

- Collect cell debris by centrifugation for 1 - 3 min at 25.000 x g and 4 °C.
- Transfer supernatant (= cleared tissue lysate) into a new, prechilled tube and discard the pellet

Store cleared tissue lysate on ice for further processing as discribed in 5.8.2. and 5.8.3. (Note: Long term storage of probes is possible at -80 °C).

5.8.2. Determination of intracellular adenosine triphosphate concentration

Familiarize yourself with the principle of the assay by reading the manufacturer manual.
The measurement of the intracellular adenosine triphosphate (ATP) concentration comprises the following three steps:

- Determination of intracellular ATP concentration
- Determination of protein concentration of the same sample
- Calculation of ATP [µg] / protein [mg]

Requirements

- ATPlite Luminescence Assay System Kit
- White flat-bottom 96-well plate (e.g. View Plate Perkin Elmer, 6005181)
- PBS w/o Ca/Mg
- Luminometer

Preparation of ATP Standard

- Reconstitute a vial of lyophilized ATP standard with water to a 10 mM stock solution (e.g. 960 µL water to solubilize 9.6 µmol ATP). The ATP stock solution is stable for several weeks at -20 °C.
- Prepare dilutions of ATP Standard in PBS with concentrations of 50,000; 10,000; 5000; 1000; 500; 100; 50 and 10 nM.
- Store the dilutions at 4 °C for a maximun of 4 weeks.

ATP measurement

Before starting the measurement, all required components of the ATPlite Kit should be equilibrated to RT. Two technical repeats are used for both, the ATP standard dilutions and the cleared tissue lysate under investigation. The substrate should be dissolved in an appropriate volume of 'substrate buffer' provided with the kit (see substrate vial).
! Reconstituted substrate solution can be stored at -20° C for longer periods of time.

The assay is being performed in a white flat-bottom 96-well plate.

- Pipet 100 µL of the cleared tissue lysate or ATP standard concentration into a respective well.
- Pipet 100 µL PBS w/o Ca/Mg in wells destined as 'blank'.
- Add 50 µL 'Mammalian Cell Lysis Solution' (provided with the kit) to all used cavities.
- Add 50 µL of the substrate solution to all used cavities.
- Let the well plate shake for 5 min in the dark on a plate shaker (450 rpm).
- Let the plate rest in the dark for 10 min without shaking.
- Start luminescence measurement and record values
- Use standard curve to assess the ATP amount in the cleared lysate.

5.8.3. Determination of protein concentration

The determination of the protein concentration is used to normalize the size of the hand cut tissue sample. subsequently the obtained ATP value is divided by the respective protein concentration.

Requirements

- Bradford Reagent
- Bovine Serum Albumin (BSA) solution (2 mg/mL)
- 96-well culture plate, clear flat-bottom (e.g. Falcon, 353072)
- PBS w/o Ca/Mg
- Photometer

Preparation of BSA protein standard

- Dilute BSA stock standard in PBS to obtain a standard curve with concentrations of 1500, 1000, 750, 500, 250, 100 and 25 µg/mL.
- Store the BSA stock solutions on ice for up to 8 h or at 4 °C after use in assay (stable for a maximum of 4 weeks).

Protein measurement

To determine the protein concentration of the cleared lysates two technical repeats are used for each sample and BSA concentration using a clear flat-bottom 96-well plate.

- Add 5 µL cleared lysate, BSA standard dilution or PBS w/o Ca/Mg ('blank') into respective wells.

- Add 250 µL Bradford reagent into each used well.
- Incubate in the dark, for 10 min at 450 rpm on a plate shaker.
- Measure absorbance (λ = 595 nm).

5.8.4. Measurement of adenylate kinase activity

Requirements

- ToxiLight™ Non-destructive Cytotoxicity BioAssay Kit
- White flat-bottom 96-well plate (e.g. View Plate Perkin Elmer, 6005181)
- ALI medium w/o Phenol red
- Luminometer

Sampling

- Sampling of cell culture medium of treated tissues is described in section 5.4.

Protocol for adenylate kinase activity (AK) measurement

- For determining enzyme activity samples and kit components are equilibrated to RT.
- Add 10 mL assay buffer to one vial of lyophilized AK detection reagent.
  - Mix gently and allow the reagent to equlibrate for 15 min at RT.
  - Use reconstituted reagent within 6 h (or within 24 h when stored at 2-8 °C).
    ! Unused reagent can be aliquotted and stored at -20 °C for up to 2 months. Once thawed, reagent shall not be refrozen and the reagent must be allowed to reach RT without the aid of artificial heat.
- Program the luminometer to perform 1 sec of integrated reading for all appropiate wells.
- Transfer 20 µL of medium samples to a cavaty of a 96-well plate. Prepare duplicate measurements of each tissue. In addition, add two wells containing fresh, unused ALI medium w/o Phenol red as reference.
- Add 100 µL of the AK detection reagent to each well.
- Incubate 5 min at RT for signal generation.
- Measure luminescence and record values.

6. Data evaluation

Two type of data are obtained with the 3D Skin Comet assay: (1) information on cytotoxicity (AK and ATP/protein) and (2) data on genotoxicity (comet results).

In order to ease processing of these amount of data a predefined excel sheet can be obtained at Phenion®. The excel files contains separate tabs for the different data types, which are automatically summarized in one graph.

6.1. Data processing

(1) Cytotoxicity data, i.e., AK and ATP/protein data of dose groups are set in reference to the solvent control. The obtained values are then used to determine the validity of these groups (please refer to section 6.2.2).

(2) Comet data are first transformed to achieve data normality and variance homogeneity. Once single comet data are summarized as medians, experiments are checked for validity before statistical significance of effects, observed in valid experiments, is investigated. Finally, the biological relevance of possible statistical flags is assessed. In detail:

Comet data from experiments are first summarized at the slide level. For each slide, a median value is calculated from the % tail DNA values measured in 50 nuclei per slide. The median is used to limit the influence of extreme values. Per tissue, these slide-level medians are averaged into one mean value. These tissue-level data are then used in all subsequent analyses.
Tissue-level data are normalized using the arc-sin square-root variance-stabilizing transformation (see formula below), where p is a proportion, rather than a percentage (i.e., divide the tissue-level % tail DNA value by 100, prior to applying this mathematical transformation of the data). The z values are then used in the statistical procedures of the prediction models.

$Z = \sin^{-1}\sqrt{p}$

6.2. Validity criteria

6.2.1. Validity criteria of an experiment

Valid experiments comprise

- A solvent control.
- A positive control.
- A negative control of untreated tissues can be omitted in case historical data obtained by the corresponding laboratory are provided to prove that the solvent has no impact on the test system.
- At least three valid concentrations of the test compound should be scored. An experiment with only two valid test groups can nevertheless be considered as valid overall, in the following cases:
    - The first two concentrations were positive and cytotoxicity was below the set thresholds for these concentrations.
    - Genotoxic effects are missing in all test groups and cytotoxicity is above the thresholds at the highest tested concentration only (because the thresholds were set to prevent false positive results).
- Three tissues per test group should be tested and evaluated.
  In exceptional cases, two tissues may be acceptable, e.g. if a substance is clearly positive in the remaining two tissues, or if a tissue is missing only at a lower concentration for a substance that does not increase % tail intensities at higher concentrations. Justification should be provided.
- The arithmetic mean of the SC cannot exceed 20% tail intensity.
- MMS as PC of standard main experiments should induce at least a 2-fold increase in % tail intensity and an absolute difference of at least 15% in comparison to the SC.
- BaP dose groups, as PC in APC experiments, should induce at least a 2-fold increase in % tail intensity and an absolute difference of at least 5% in comparison to the SC/APC group.

6.2.2. Validity criteria of a dose group

- Three tissues per test group should be tested and evaluated (for exemptions see 6.2.1).
- Comets from at least 2 slides should be analyzed for each test group.
- At least 100 comets per tissue (e.g. 50 comets per slide) should be analyzed for each tissue.
- Following thresholds of cytotoxicity are used:

  (1) If the mean of three tissues shows a 2-fold increase (or higher) in AK release in comparison to the mean of the concurrent SC this test group is classified as cytotoxic and it is excluded from the assessment of the genotoxic potential of a test compound.

  (2) If the mean of three tissues shows a decrease of 50% (or more) of the ATP/protein ratio in comparison to the ratio of the concurrent SC this test group is classified as cytotoxic and it is excluded from the assessment of the genotoxic potential of a test compound.

If cytotoxicity is seen with both measures the more sensitive parameter is used. Nevertheless, in some cases a decrease in analyzable nuclei (comets) on the slides might be the only indication of strong cytotoxicity. Therefore, the reason for considering a dose strong cytotoxic needs to be recorded for each experiment.

6.3. Statistical evaluation

First an ANOVA is performed. Only in case of significant concentration effects, the single test concentration will be compared to the SC using Dunnett's multiple comparison (Dunnet, 1955). Because decreases in the % tail DNA are not of primary interest, the pair wise comparisons will be one-sided (testing for increase). In case a clear decrease of fluorescence intensity is observed in an experiment the two-sided comparison will be applied.

I. An experiment is considered positive for genotoxicity if it has several concentrations that produce a dose-related increase and at least one statistically significant increase in the percentage of tail DNA compared to the solvent control at concentrations that do not exceed the limit of cytotoxicity.

II. A test substance is called positive for genotoxicity overall if only one concentration produced a statistically significant increase in percentage of tail DNA and the significant effect was reproduced in an independent experiment.

A positive call in one cell type is sufficient to consider a regular or APC experiment as positive. In case neither of the criteria are fulfilled, the test substance is considered as negative. In case some criteria are fulfilled the study is considered equivocal and needs further investigation.
It may be necessary to repeat an experiment once more to come to a final conclusion.

6.4. Consideration of the biological relevance

In addition to the statistical criteria defining a positive or negative call the following three criteria needs to be fulfilled in addition to also consider the biological relevance of an effect:

- The response has to be dose-dependent.
- At least one dose needs to be statistically significant different from the SC.
- At least one test group needs to be outside the historical control data range (lab specific: mean of SC of the last 15 experiments plus 2σ).

7. References

Bätz FM, Klipper W, Korting HC, Henkler F, Landsiedel R, Luch A, von Fritschen U, Weindl G, Schäfer-Korting M. Esterase activity in excised and reconstructed human skin--biotransformation of prednicarbate and the model dye fluorescein diacetate. Eur J Pharm Biopharm. 2013 84(2):374-85.

Brinkmann J, Stolpmann K, Trappe S, Otter T, Genkinger D, Bock U, Liebsch M, Henkler F, Hutzler C, Luch A. Metabolically competent human skin models: activation and genotoxicity of benzo[a]pyrene. Toxicol Sci. 2013 131(2):351-9.

Dunnett WC. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc. 1955 50:1096-1121.

Hoffmann S, Reisinger K, Brinkmann J, Down T, Fischer A, Haase A, Henkler F, Liebsch M, Luch A, Petrick C, Pirow R, Reus A, Said A, Schäfer-Korting M, Schulz M, Pfuhler S. 3D Skin Comet assay: Genotoxicity assessment addressing the dermal route of exposure. 10th World congress on alternatives and animal use in the life science, 2017.

Jäckh C, Blatz V, Fabian E, Guth K, van Ravenzwaay B, Reisinger K, Landsiedel. Characterization of enzyme activities of Cytochrome P450 enzymes, Flavin-dependent monooxygenases, N-acetyltransferases and UDP-glucuronyltransferases in human reconstructed epidermis and full-thickness skin models. Toxicol In Vitro. 2011 25(6):1209-14.

Mewes KR, Raus M, Bernd A, Zöller NN, Sättler A, Graf R. Elastin expression in a newly developed full-thickness skin equivalent. Skin Pharmacol Physiol. 2007 20(2):85-95.

OECD Guidelines for the Testing of Chemicals, Test No. 471: Bacterial Reverse Mutation Test. 1997. http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=4ld1df8mkacg.x-oecd-live-02

OECD Guidelines for the Testing of Chemicals, Test No. 489: *In Vivo* Mammalian Alkaline Comet Assay, 2016. http://www.oecd-ilibrary.org/docserver/download/9716431e.pdf?expires=1500387720&id=id&accname=guest&checksum=2DA633C5AAE1F2191453305978B2207F

Ostling O, Johanson KJ. Micorelectrophoretic study of radiation-induced DNA damaged in individual mammalian cells. Biochem Biophys Res Commun. 1984 123: 291-8.

Reisinger K, Blatz V, Brinkmann JP, Downs TR, Fischer A, Henkler F, Krul C, Liebsch M, Luch A, Pirow R, Reus AA, Schulz M, Pfuhler S. Validation of the 3D Skin Comet assay using full thickness skin models: transferability and reproducibility. Mutat Res 2018 827:27-81.

Reus AA, Reisinger K, Downs TR, Carr GJ, Zeller A, Corvi R, Krul CA, Pfuhler S. Comet assay in reconstructed 3D human epidermal skin models--investigation of intra- and inter-laboratory reproducibility with coded chemicals. Mutagenesis. 2013 28(6):709-20.

SCCS, Scientific Committee on Consumer Safety. OPINION ON N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (B34) Submission IV. 2016. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_196.pdf

SCCS (2015) Scientific Committee on Consumer Safety. Opinion on 2,6-Dihydroxyethylaminotoluene COLIPA no A138. SCCS/1563/15. Adopted on 25 June 2015. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_188.pdf.

SCCS (2014a) Scientific Committee on Consumer Safety. Opinion on Basic Brown 17 COLIPA no B007. SCCS/1531/14. Adopted on 24 March 2014, revision of 18 June 2014. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_152.pdf.

SCCS (2014b) ADDENDUM to the SCCS's Notes of Guidance (NoG) for the Testing of Cosmetic Ingredients and their Safety Evaluation, 8th Revision (SCCS/1501/12). http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_156.pdf.

Singh NP, McCoy MT, Tice RR, Schneider EL.; A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988 175:184-91.

Speit G, Schütz P, Hoffmann H. Enhancement of genotoxic effects in the comet assay with human blood samples by aphidicolin. Toxicol Lett. 2004 153(3):303-10.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000 35:206-21.

Wiegand C, Hewitt NJ, Merk HF, Reisinger K. Dermal xenobiotic metabolism: a comparison between native human skin, four in vitro skin test systems and a liver system. Skin Pharmacol Physiol. 2014 27:263-75.