

**RECONSTRUCTED SKIN MICRONUCLEUS ASSAY (RSMN)
using the EpiDerm™ human skin model**

PROTOCOL

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DISCLAIMER

This protocol is intended for users with practical experience in in vitro genotoxicity testing only. Therefore, it is assumed that users will be aware of the respective national or regional regulations that they have to comply with. It is also assumed that they know the risks in handling (incl. disposal) hazardous substances and how to mitigate them.

1. Purpose

The purpose of the RSMN is to evaluate the potential dermal genotoxicity of the test article to the EpiDerm™ Skin Model after repeat exposure to the test article for 48 and/or 72 hours. Cytotoxicity of test articles will be determined by measuring the relative percent binucleation and the relative live cell count of the exposed cultures compared to the solvent control, (whichever toxicity method is more sensitive). Genotoxicity potential will be determined by measuring the frequency of micronucleated binucleated cells in the test article treated cultures compared to the solvent control for statistical significance.

2. Test system

The RSMN is based on the MatTek Corporation's EpiDerm™ Skin Model. The features that make the EpiDerm™ Skin Model advantageous in the study of potential dermal toxicity are:

- it is derived from human skin
- the target cells are epithelial
- the tissue has a functional *stratum corneum*
- the test materials are applied directly to the tissue surface, at air interface so that undiluted and/or end use dilutions can be tested directly.

3. Materials

- EpiDerm Tissues: MatTek EPI-200-MNA kit, includes NMM (new maintenance medium)
- Mitomycin C (MMC): Sigma, M4287
- Cytochalasin B (CytoB): Sigma, Cat# C 6762
- Calcium/Magnesium-Free Dulbecco's Phosphate Buffered Saline (DPBS): MatTek (or equivalent)
- Trypan Blue, Sigma, Cat# T8154 (or equivalent) or other cell count method
- EDTA 1g/L (~2.7mM), Quality Biological (or equivalent)
- KCl (0.075M), Sigma, Cat# P9327 (or equivalent)
- Acridine Orange Solution (10 mg/mL), Sigma, A8097. Final concentration of AO stain is 40 µg/mL (1 mL of stock in 249 mL of DPBS). Store stained slides (indefinitely) and unused stain (up to four weeks) refrigerated and protected from light.
- Trypsin (0.25%) – EDTA (0.02%), JRH Biosciences, Cat# 59228-100M (or equivalent)
- Cell culture media (DMEM containing 10% FBS and 1% L-glutamine) (Quality Biological or equivalent)
- Methanol, Sigma, Cat# 270474 (or equivalent)/ Glacial Acetic Acid, Sigma, Cat# A-6283 (or equivalent) mixed at 3:1 ratio (volume per volume). Mix fresh on morning of harvest and keep cold.
- Microslides, 25X75 mm, 1mm thick, Goldseal or (VWR Cat# 48312-002 or equivalent)
- 12-well plate, Falcon, Cat# 3043 (or equivalent)
- Polypropylene 15 ml conical tube
- Polypropylene 50-ml conical tube
- Fine Forceps
- Centrifuge

4. Preparation and application of test article

Prepare the test article concentrations in the solvent identified in the solubility study (see below), fresh on each day of dosing. Topically apply ten microliters of test the article concentration to the middle of the EpiDermTM tissue. Rotate the plate containing tissues immediately following the test article application to ensure a full coverage of the topical surface of the tissue.

5. Preparation and application of the vehicle and positive control

Mitomycin C (MMC) is the positive control. A master (stock) concentration of the positive control is prepared and frozen.

Dilute the MMC positive control in acetone and apply a ten microliter dose directly to the EpiDerm™ tissue. Rotate the plate containing tissues immediately following the positive control application to ensure a full coverage of the topical surface of the tissue. Apply a second ten µl dose approximately 24h after the first dose. Apply a third ten µl dose approximately 24h after the second dose for the 72 hour exposure assay.

Apply two to three ten microliter doses of the recommended vehicle control (VC), spaced 24h ± 3h apart, directly to the EpiDerm™ tissue directly on the EpiDerm™ tissue. Tilt the plate containing tissues immediately following the negative control application to ensure a full coverage of the topical surface of the tissue.

Mitomycin C Master Stock Positive Control preparation

Make a 0.5mg/mL stock solution of MMC by adding 4.0 ml of room temperature, sterile, tissue culture grade water to the vial containing 2 mg of MMC. Vortex until complete solubility is achieved. Using a calibrated micropipette, dispense a volume of MMC stock into sterile labeled cryovials that will allow dilutions to be easily made to achieve 3µg/mL. Cap each vial tightly and store aliquots at -15 to -25°C. Each lab should follow the positive control performance carefully and consider a new preparation when past the manufacturer's expiration date or with low MN frequency.

On the day of use, remove a vial of MMC stock from the freezer and bring it to room temperature, do not thaw in a 37°C water bath. Vigorously vortex the thawed aliquot of MMC until all dark purple flecks (precipitates) are dissolved. If dark flecks are still visible after vortexing, the vial may be briefly sonicated. Make the "Initial dilution" of 100µg/ml (see example chart) by adding acetone to the vial. Mix well, and check again for dark precipitate. If the dark precipitate persists, discard the aliquot and thaw another, or make a fresh batch of MMC stock. Expect to see some cloudiness in the first dilution of 10µg/ml, however if an easily visible chunky white precipitate forms, discard the dilution and start over with a different aliquot of MMC stock.

Example of MMC dose preparation scheme for the Micronucleus assay:

- Begin with a vial containing 100µg MMC stock (200 µL MMC stock x 0.5 µg/µL)
- Initial dilution: Add 0.8 mL of acetone to the stock vial = 100 µg/mL
- Dilution 1: 0.5 mL of "Initial" diluted with 4.5 mL of Acetone = 10 µg/mL
- Dilution 2: 1.5 mL of "Dilution 1" diluted with 3.5 mL of Acetone = 3 µg/mL
- Prepare the MMC dose(s) fresh each day of dosing the tissues.

6. Cytochalasin B preparation

Expose the cultures to NMM containing 3 µg/mL Cytochalasin B (CytoB) for the duration of the assay. Prepare and freeze a master (stock) concentration of CytoB.

3.3 mg/mL Master CytoB Preparation

Add 3.3 mL DMSO to a 10 mg vial of Cytochalasin B and vortex until completely solubilized. Using a calibrated micropipette, aliquot 100 µL (or other appropriate volume) of CytoB into appropriately labeled cryovials. Cap tightly and store at -15 to -25°C. The expiration date will be 1 year or less based on the expiration date specified for CytoB.

Thaw a 3 mg/mL aliquot of Cytochalasin B on each day of dosing. Prepare fresh CytoB NMM by adding 1 µL of 3 mg/mL CytoB stock per mL NMM, the final concentration is 3 µg(cytoB)/mL NMM.

7. Receipt of EpiDerm™ kits

Store the EpiDerm™ Skin Model kit and included solutions as indicated by the manufacturer.

Label sterile 6-well plates to indicate treatment for each tissue. Warm the NMM to approximately 37°C and dispense one mL into appropriate wells of labeled 6-well plates.

Prior to opening the inner sealed package containing the 24-well plate of tissues, and while holding the plate of tissues in such a way that the bottom of the tissue can easily be seen and inspect each EpiDerm™ tissue in its respective cell culture insert for air bubbles between the agarose gel and the insert. Do not use cultures with air bubbles (air pockets) greater than 50% of the insert area. Open the inner package and further inspect the tissues for obvious defects on the tissues such as blisters, raised edge (possible tissue detachment), and excess moisture on the tissue. Make a note of the condition and location of the tissue. Some clear to yellowish droplets thought to be due to condensation are often seen on the surface of the tissues especially during hot weather. These droplets may be carefully blotted if desired but will often resolve when the tissues are left to recover overnight in the incubator. Tissues often have what appears to be dark red dried media, or red liquid media inside the insert around the periphery of the tissue. If there is enough moisture that it moves when the tissues are at a slant, the moisture may be removed by gentle blotting with a sterile swab or by extremely gentle vacuum aspiration.

Using forceps, carefully transfer an appropriate number of EpiDerm™ tissues in their inserts from the 24-well shipping tray into the labeled 6-well plates. Incubate the EpiDerm™ inserts at 37±1°C in a humidified atmosphere of 5±1% CO₂ in air (standard

culture conditions) overnight, or at least 1 hour for the 72 hour exposure assay. Do not use tissues in the assay that are defective **prior** to dosing. Dosing solutions may affect the health of the tissues resulting in detachment (raised edges), moisture around the periphery (leakage) or blistering on the surface of the tissue, etc. Continue dosing those tissues.

8. Experimental design and methodology

The experimental set-up of the RSMN follows the approach of classical in vitro genotoxicity test methods and comprises three steps:

- Solubility determination (section 4.1)
- Dose range finding experiment (section 4.2)
- Definitive testing (section 4.3)

8.1. Solubility study

The aim of this first step is to identify the highest soluble concentration (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 at 10% (w/v), i.e. 100 mg/mL. 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 the highest soluble concentration.

Acetone (10 µl/tissue) is used as the standard solvent. Ethanol, 70/30% ethanol/water and 4:1 acetone/olive oil are also acceptable alternatives, however DMSO is not considered appropriate. Saline seems to work for the 48h protocol, but limited experience exists for its use under 72h conditions. For test articles that have been evaluated in standard in-vitro mammalian cell genotoxicity assays, a dose range of around 200-fold higher than the concentrations shown to be toxic/genotoxic in vitro may be useful to start with in the RSMN.

Precipitation of test compounds on the surface of tissues shall be avoided as it potentially leads to false-positive results. The tissue surface is investigated for precipitation, i.e., any test compound residue at each subsequent dosing with the naked eye.

8.2. Dose range finding experiment

The main objective of the dose range finding experiment (DRF) is to determine the cytotoxicity of a test article to inform the selection of the doses for the main experiment. A 48 hour exposure dose range finding assay will be done to establish an appropriate test article dose range for the definitive assay. At least one tissue will be tested for each test article dose. The

highest concentration of the test article will be prepared in the identified solvent. All test article dilutions will be prepared from the highest prepared concentration.

8.3. Definitive testing

Definitive testing will be performed exactly like the dose range finding assay with the exception that typically triplicate tissues will be tested for each test article dose. Doses should include concentrations that reduce relative percent binucleation and/or relative live cell counts by $50 \pm 10\%$ (high cytotoxicity), $30 \pm 10\%$ (intermediate cytotoxicity), and $10 \pm 10\%$ (low cytotoxicity), unless the test article showed insignificant cytotoxicity up to the highest prescribed test article concentration. For the high cytotoxicity dose group, to qualify for micronucleus scoring, there must be at least two tissues per concentration that fulfill this criterion. Precipitation of the test article on the surface of the tissue is recorded in the beginning and at the end of treatment, and the lowest precipitating dose will be the highest concentration that qualifies for micronucleus scoring. Results from concentrations higher than the lowest precipitating dose will not be considered in the assessment as remaining test material on the tissue surface has been shown to impact the tissue quality. The occurrence of liquid or oil on the surface of tissues will not be considered precipitation, but should be recorded.

On the starting date of the assay, (following an overnight incubation of the EpiDerm™ inserts for 48 hr treatment and at least 1 hr incubation for 72 hr treatment), prepare and warm a sufficient volume of NMM with 3 µg/mL CytoB to ~37°C to use for the day. Prepare dilutions (maximum spacing one half) of the test article in the indicated room temperature solvent. Make dilutions of test articles and controls fresh on each day of dosing. Visually observe all tissues and record observations prior to dosing. Exclude all tissues with significant defects such as blisters, detachment and significant moisture.

Refeed the tissues with fresh, warm NMM containing 3 µg/mL CytoB (two possible methods: aspirate media under the well and add 1 mL of fresh media with CytoB or prepare new 6-well plates with fresh media and transfer tissues from the original 6-well into newly prepared 6-well). Apply ten µL of the test article or control to the surface of tissue, rotating plate to insure complete coverage. Incubate the cultures at standard culture conditions.

24 hours \pm 3h after the first dosing, replace the medium with fresh NMM containing 3 µg/mL Cytochalasin B. Visually observe all tissues and record observations. Prepare fresh controls and dilutions of the test article in the identified solvent and dose the cultures a second time. Incubate at standard culture conditions for approximately 24 hours. Apply the second dose of test article to tissues that develop blisters, leakage or precipitates after being dosed, as these defects may be caused by the test article.

For the tissues in the 72 hour treatment, replace the medium with fresh NMM containing 3 µg/mL Cytochalasin B 24 hours \pm 3h after the second dosing. Visually observe all tissues

and record observations. Prepare fresh controls and dilutions of the test article in the identified solvent and dose the cultures a third time. Incubate at standard culture conditions for approximately 24 hours. Apply the test article to tissues that develop blisters, leakage or precipitates after being dosed, as these defects may be caused by the test article.

9. Termination of treatment and trypsinization

Harvest the tissues 48 ± 3 or 72 ± 3 hours after the initial test article exposure. Visually observe all tissues and record observations. The tissues will be harvested (trypsinized) 4-6 tissues at a time. Keep the remaining tissues at standard culture conditions until harvest.

A. First Wash

In the wells of the left half of the labeled 12-well plate, dispense ~5mL of room temperature DPBS. Using forceps, remove a tissue insert from the treatment plate and blot the bottom on a paper towel to remove excess medium. Place the tissue insert into a well containing DPBS and completely immerse the tissue for 5-15 minutes. Work with a maximum of 6 tissues per technician at a time.

B. Second Wash

In the remaining 6 wells of the same 12-well plate dispense ~ 5 mL of room temperature 0.1% EDTA solution. Using forceps, remove the tissue insert from the first wash and decant DPBS by inverting the insert. Blot insert to remove excess DPBS, then completely immerse the tissue insert in the correct well containing EDTA solution for ~15 minutes

C. Trypsinization

Dispense 1ml of warm (~37oC) Trypsin-EDTA solution into each of six wells in a labeled 12-well plate. Using forceps, remove the tissue insert from the second wash and decant EDTA by inverting the insert. Blot insert to remove excess EDTA, then the tissue insert in the designated well containing trypsin. Dispense an additional 0.5 mL of warm Trypsin-EDTA solution inside the tissue insert. Incubate the tissues at 37oC for 10-15 minutes.

D. Dislodge Cells

After 15 min exposure to trypsin, the tissue with 0.5mL trypsin inside the insert, is transferred to a new well containing 1 ml of fresh trypsin. While holding the insert with forceps in one hand, fine forceps are used in the other hand to gently grasp the edge of the tissue, peel it free, and place it in the well containing the fresh trypsin. Do not remove trypsin-EDTA inside the cup.

(Note: Alternative method: tissues are removed from the insert as described above and placed into the original trypsin well with insert placed back into the well on top of the

tissue. One mL of neutralization medium is added directly into the insert and well to stop the trypsin. Using this method, after cells are dissociated (section X.E. below), they are collected into empty labeled 15ml centrifuge tubes)

E. Cell Collection

Using a P1000 pipettor (or a 2 ml serological pipet) rinse the inside of the insert thoroughly (4 to 6 times) to collect all of the previously adherent basal cells. Direct the stream to four sides of the surface (north south east and west) and once in the center, transfer the cell suspension to the well containing the tissue and discard the insert. Using the same pipet, agitate the detached tissue to loosen any additional cells (about 4 or 5 times). Aspirate the cell suspension (~1.5 mL) being careful to exclude the tissue, and transfer it to a labeled 15ml centrifuge tube containing 1ml neutralization medium. *(Note: If using the alternative method, the trypsin will already be neutralized and cell suspension will be transferred to an empty appropriately labeled tube.)*

Mix well and transfer a volume of the neutralized cell suspension into pre-labeled vials for viable cell counts. Cell counts should be done as soon as possible.

Centrifuge the cell suspensions at 100XG for 5 min. at room temperature.

10. Fixation and slide preparation

KCl must be pre-warmed to approximately 37oC

After centrifuging, carefully aspirate as much of the medium as possible without sacrificing the cell pellet. Agitate to loosen the cell pellet. Keep the cells in suspension while adding the warm KCl drop by drop into the tube while holding it on a vortex set on set at approximately 550 rpm. If a mixer is not used, very slowly add 1 ml of warm (~37oC) KCl, keeping cells in suspension by occasionally flicking the tube with a finger. Incubate at RT for three minutes.

After three minutes, slowly (should take ~10 seconds) add 3 mL of cold (prepared fresh on the day of use) MeOH/Acetic Acid (3:1,v/v) and mix gently. Centrifuge the cell suspension at 100G for five minutes.

Label slides with the study number, tissue number, and other necessary identification prior to dispensing fixed cells.

Note:

A second fixation/centrifugation step using MeOH/Acetic Acid (40:1or other), may be done to reduce the amount of salt crystals that are sometimes problematic. If slides cannot be dropped (cells dispensed) on the day of harvest, the fixed cells may be stored in 99:1

fixative (MeOH/Acetic Acid (99:1,v/v) at 2-8oC for a few days, then centrifuged and dropped on slides.

Aspirate the fixative down to ~40-60µL. Loosen the cell pellet and gently agitate the fixed cells, aspirate the cell suspension and using a glass Pasteur pipet, dispense a 15 to 20 µL drop directly onto the pre-labeled micro slide (*Note: dispensing a 20-30µl drop using a 100µL Ranin LTS pipettor works well to spread cells when using 40:1 fix*). Do not overlap cell drops on the slide. If possible, prepare two slides per tissue, one to be scored and one as the backup. Allow slides to air-dry room temperature.

11. Slide staining and scoring

The relative live cell count data may be analyzed before the assay qualification check using AO stained slides is done. An assay must have a minimum of three valid concentrations (high, intermediate, and low toxicity), with a minimum of two valid tissues per concentration.

Once slides are completely dry, immerse them in room temperature AO solution for two minutes. Rinse in DPBS three times, one minute each rinse. Air dry at room temperature and store @ 2—8o protected from light until scored.

After staining, perform an assay qualification check by evaluating one each of the vehicle and positive control slides to determine if the assay meets the % BN for the vehicle control requirement, and that the number of micronucleated binucleated cells in at least 500 binucleated cells meets or exceeds the historical average MNBN of the positive control for the lab performing the assay. If the assay qualification check is acceptable, the slides are scored for BN.

Based on the relative % BN and observations made during the assay (precipitation - lowest precipitating concentration may be evaluated for MN) appropriate slides are selected for MN frequency counting.

Use the binucleated cells and the micronucleus scoring guidelines by Dahl et al, Mutation Research 720 (2011) 42–52 as the scoring criteria. Evaluate the toxicity where tissues with less than 60% survival by relative binucleation, or relative viable cell count when compared to the vehicle control, are considered toxic and will not be scored for micronuclei but averaged in to calculation of cytotoxicity.

The vehicle and positive control slides that were used in the qualification check are coded and added the slides for analysis. If slides have not already been coded by a neutral party, they should be coded before being evaluated for MN (scored for MN) so that a blind scoring can be performed.

12. Data analysis

Relative Viable Cell Count Determination:

Using trypan blue exclusion or other live vs. dead cell count method, calculate the relative viable cell count for each tissue. Either the relative viable cell count or the relative binucleation, whichever is more sensitive, is used to select the slides for micronucleus scoring. The % relative cell count is calculated according to the following formula:

% Relative Viable Cell Count (RVCC) =

Viable Cell count (x10⁵) the tissue X 100/ average viable cell count (x10⁵) of the solvent controls

Cytotoxicity based on cell count = 100-% Relative Viable Cell Count (RVCC)

T = Treated tissue

C = Control tissue

% Binucleation and Relative Binucleation:

The fluorescence microscope will be used to determine the percent binucleation and the micronucleus frequency of each slide. 20 to 40X objectives in “dry” condition will be used for scoring for binucleation.

The % binucleation is determined based on at least a 500 cell count. If fewer than 500 scorable cells are available for determining binucleation, then the slide will not be evaluated for MN and will be considered 0% survival (100% cytotoxic) and averaged into the calculation of the cytotoxicity for the test article concentration. Count the mononucleated, binucleated, and multinucleated (> 2 nuclei) cells, and use the following calculation to determine the % Binucleation of each slide:

% Binucleation of the slide = number of binucleated cells x 100/ (# 1N + # 2N + # >2 nucleated cells)

Use the following calculation to determine the % relative binucleation for each slide:

% Relative Binucleation of the slide =

% Binucleation of the Treatment slide X 100/ average % Binucleation of solvent controls

Cytotoxicity based on % BN cells = 100 - % Relative Binucleation of the slide

Selection of dose levels for the evaluation of micronucleus:

At least three valid doses from definitive and confirmatory micronucleus assays will be evaluated for micronuclei using following criteria:

- i. For cytotoxic test article, the highest valid dose will be 50 to 60% cytotoxicity.
- ii. For the test article that precipitates on the tissue surface at the end of treatment, the lowest precipitating dose level will be highest valid dose level for MN analysis.
- iii. If the test article is non-cytotoxic, at least three doses including the highest prescribed will be included.

Percent Micronucleus Determination:

The percent micronucleus is determined based on at least 500 (typically 1000) binucleated (BN) cells/tissue. Tissues with < 500 BN cells are considered unscorable. The MN scoring is conducted separately from the % BN count. Count the number of binucleated cells with and without micronucleus. Use 40 to 60X objectives in “dry” condition for the MN slide scoring.

Use the following calculation to determine the % micronucleus of a slide:

% micronucleus = # of BN cells with at least one micronucleus X 100/ Total # of binucleated cells

13. Validity criteria

A number of validity criteria have been defined to ensure that only valid experiments are used in the assessment of a substance.

- The yield of viable cells in the vehicle control should be higher than 5×10^4 cells per tissue. Any vehicle treated tissue with less than 5×10^4 cells per tissue is eliminated. If less than two tissues meet the criterion, the entire experiment is not valid.
- The binucleation rate in each vehicle control tissue must be at least 25%. Any control tissue with < 25% binucleation in the vehicle is eliminated. If less than two tissues meet the criterion, the entire experiment is not valid.
- The positive control must cause a statistically significant increase in the % MN compared with the average of the vehicle control tissues (one sided fisher's exact $p < 0.05$). If not met, the entire experiment is not valid.

- Controls and at least three concentrations* of the test article must meet the criteria below:
 - Controls and each valid concentration will have at least two tissues per treatment.
 - 500 total cells (minimum) per tissue are analyzed for proliferation (1N, 2N, $\geq 3N$). Any tissue with < 500 cells is considered 100% toxic and averaged into the group toxicity.
 - The % relative viable cell count is 40% or more in each test article-treated tissue (no more than $55\% \pm 5\%$ toxicity) compared to the average of vehicle control tissues.
 - The % MN in the solvent controls (average) should not exceed the historical vehicle control range for the testing lab.

*An experiment with only two valid dose-groups can be considered a valid experiment if one or both of the concentrations was clearly positive and cytotoxicity was acceptable.

Note that in case the vehicle or positive control fails to meet any criterion, the entire experiment is not valid.

14. Assessment

Statistical analysis will employ a Fisher's exact test for pair-wise comparisons between each treated and vehicle control treatment groups. The Cochran-Armitage trend test will also be conducted. One-sided tail probabilities for an increase or positive trend will be used to evaluate statistical significance ($p < 0.05$).

Positive or Negative results, both for test articles and experiments, are determined according to the following criteria:

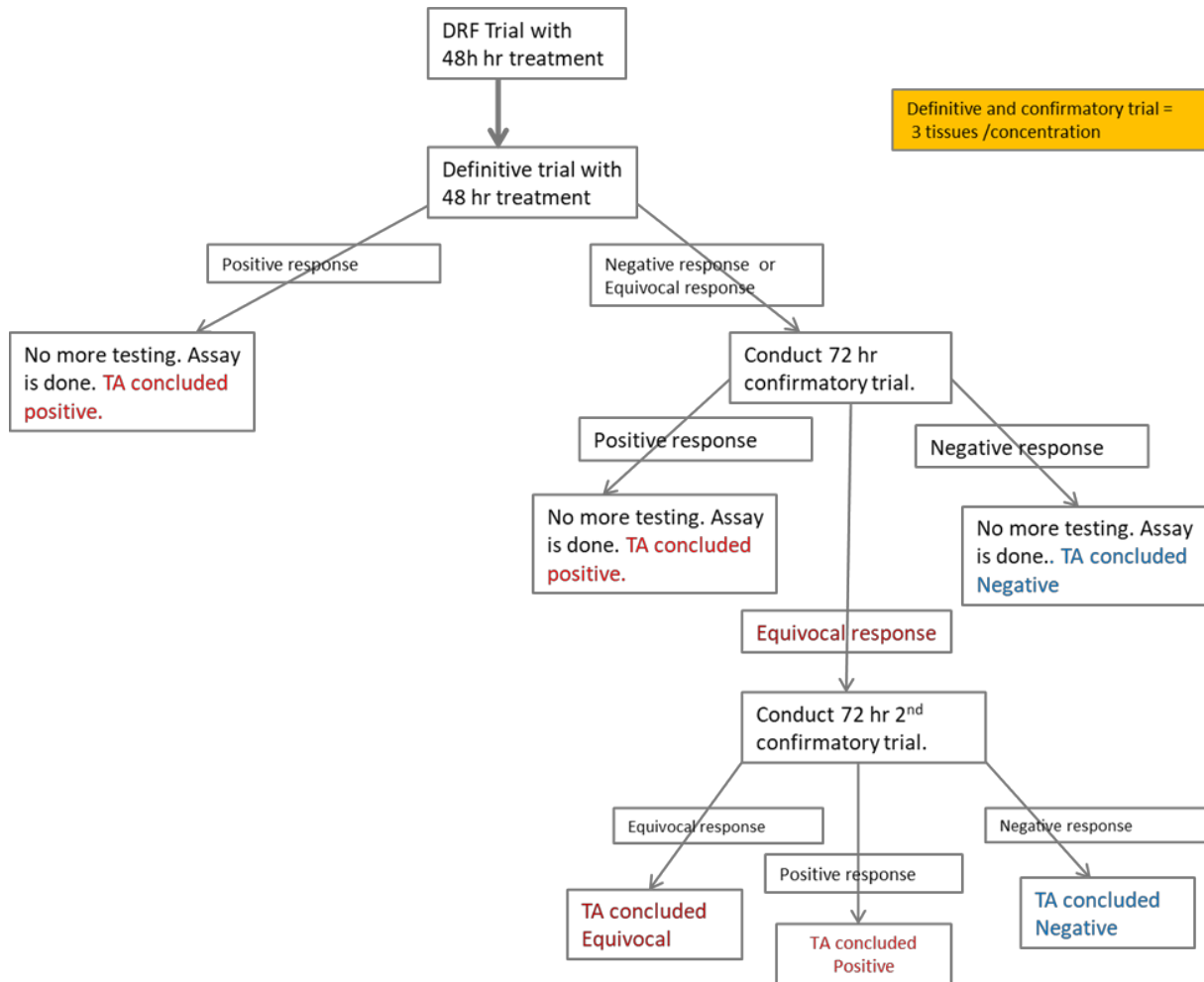
Assessment of an experiment:

- Results from concentrations higher than the lowest precipitating dose will not be considered in the assessment as remaining test material on the tissue surface has been shown to impact the tissue quality.
- An experiment is considered positive if it has one or more concentrations that are statistically significant (experiment has to meet validity criteria) and outside the upper 95% confidence limit of the historical control data. When these criteria are met, the Cochran– Armitage trend test ($p < 0.05$) will be used in the overall judgment of the response.
- An experiment is considered negative if no statistically significant or dose dependent increases in the frequency of MN-BN are observed
- If the results of an experiment do not meet the criteria of a clear positive or negative (as defined above), it will be considered equivocal. If a data point that is statistically significant but is below the 95% confidence limit of the historical control data the experiment is considered equivocal in this experiment.

Assessment of a test article:

- Positive call - A test article is called positive overall if it has at least one experiment with two or more statistically significant concentrations, or one concentration that is statistically significant (one sided Fisher's Exact test) and biologically relevant as defined below in one independent study.
- Negative call - A test article is called negative overall if in two independent studies (one 48 hour exposure and one 72 hour exposure assay) the test article produces no statistically significant or biologically relevant increase in the frequency of MNBN
- Equivocal call - Despite extensive testing, a test article may produce results that are neither clearly positive nor clearly negative. In those rare instances the test article may be considered to have produced equivocal responses.
- Biological relevance of the findings will be considered, and will take into account the above mentioned points (dose-dependence, strength of the effect in relation to the 95% CI of the historical vehicle control data) as well as reproducibility between tissues within the experiment.

Flow chart 1: Schematic representation of the experimental layout of the validation experiments. Please note that until now (May 2016; Validation phase Phase 2c) the confirmatory trial has been performed using the 48h treatment protocol.



15. Acknowledgement

This protocol evolved over several years. During this time, too many researchers provided value contributions to be acknowledged individually. Therefore, please refer to the references, especially Pfuhler et al., 2021 (Mutagenesis, 36(1):1-17. doi: 10.1093/mutage/geaa035).