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The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: Detailed protocol and harmonized scoring atlas

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ABSTRACT

The European Cosmetic Toiletry and Perfumery Association (COLIPA), along with contributions from the European Centre for the Validation of Alternative Methods (ECVAM), initiated a multi-lab international prevalidation project on the reconstructed skin micronucleus (RSMN) assay in EpiDerm™ for the assessment of the genotoxicity of dermally applied chemicals. The first step of this project was to standardize the protocol and transfer it to laboratories that had not performed the assay before. Here we describe in detail the protocol for the RSMN assay in EpiDerm™ and the harmonized guidelines for scoring, with an atlas of cell images. We also describe factors that can influence the performance of the assay. Use of these methods will help new laboratories to conduct the assay, thereby further increasing the database for this promising new *in vitro* genotoxicity test.

1. Introduction

As a result of the 7th Amendment of the Cosmetics Directive [1], *in vivo* genotoxicity assays have been banned as of March 2009 for safety testing of cosmetics and cosmetic ingredients in the European Union. *In vivo* genotoxicity studies are also impractical for large-scale chemical evaluation programs such as REACH [2]. Currently available *in vitro* genotoxicity assays have an unacceptably high rate of false positive results that are not confirmed by *in vivo* genotoxicity tests or carcinogenicity assays [3,4]. Relying solely on *in vitro* assays would thus result in the removal of potentially valuable chemicals early in product development. A reconstructed skin micronucleus assay (RSMN assay) in EpiDerm™ models was developed [5–7] as a potential replacement for the *in vivo* micronucleus assay, and is especially relevant for chemicals for which human exposure is expected to be dermal. Recently, the European Cosmetic Toiletry and Perfumery Association (COLIPA), with contributions from ECVAM, initiated an international prevalidation project to evaluate genotoxicity assays by use of 3D reconstructed human skin, including the RSMN assay [8], to support the evaluation of chemicals to which humans are exposed dermally, including cosmetics ingredients. Good intra- and inter-laboratory reproducibility of the RSMN in EpiDerm™ between laboratories in the United States and Europe was observed [8], supporting the conclusion that the RSMN assay is a valuable *in vitro* method for genotoxicity assessment of dermally applied chemicals. The use of the RSMN assay in the genotoxicity assessment of cosmetics was recently described [9].

Following the modular approach to validation practiced by ECVAM [10], the first step of the project was to standardize the protocol and successfully transfer the assay to laboratories that had not performed the assay previously. Earlier reports describe the transfer of the method between laboratories in the United States [6,7]. The next focus of the COLIPA project was on transfer and harmonization with laboratories in Europe [8]. Two training workshops were held at the Institute for In Vitro Sciences (IIVS) to standardize the protocol and harmonize scoring of micronuclei.

Here, we describe the standardized protocol for the RSMN assay that resulted from the training workshops, pointing out key aspects that can impact the assay as well as describing the statistical

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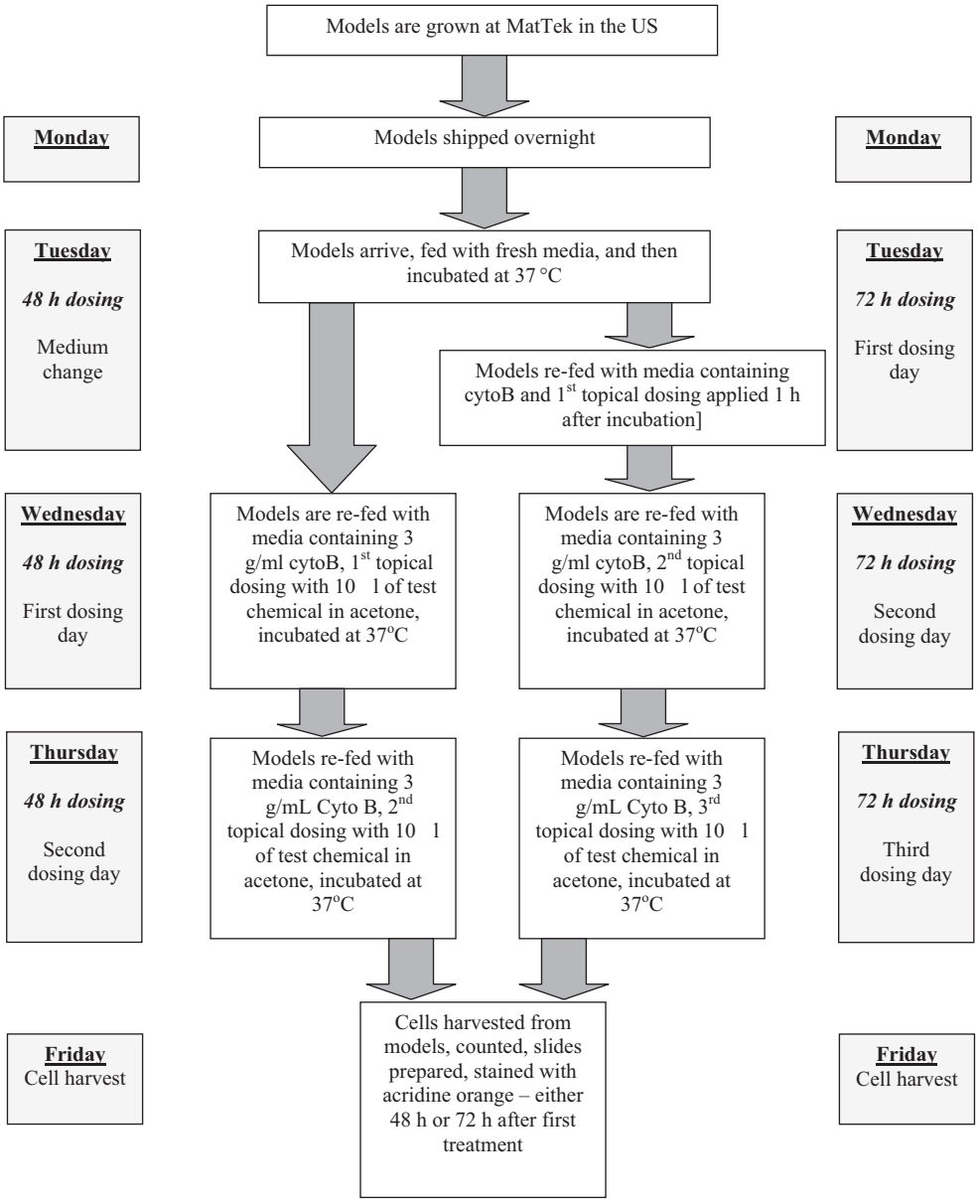

Fig. 1. An overview of the RSMN-assay timeline (as used by others for pre-validation studies [6–8]).

methods recommended for data analysis. We also present detailed guidelines for scoring, along with an atlas of cell images. Use of these methods by new laboratories will ease their adoption of the assay, and increased use of the assay by new laboratories will add to our understanding of the predictability of this promising new *in vitro* genotoxicity test.

2. Methods and discussion

2.1. General time line and methodology

An outline of the assay is shown in Fig. 1. EpiDerm™ models are shipped overnight from the MatTek Corporation (Ashland, MA, USA) on Mondays. Typically, models shipped within the US arrive on Tuesday morning and models shipped according to special shipment conditions to Europe arrive late on Tuesday afternoon. Once the models arrive they are transferred from an agarose-coated 24-well plate to a 6-well plate containing fresh medium. The models are then placed in an incubator overnight. On Wednesday, the medium is replaced with medium containing cytochalasin B (cytoB). On the same day, the first dose of test compound (normally in acetone) is applied to the upper side of the skin model. On Thursday, the medium is again replaced with fresh medium containing cytoB and the model is treated with a second dose of test compound. On Friday, after 48 h of exposure, cells from the basal layer and *stratum spinosum* of the models are harvested and prepared for slide analysis as described by Curren et al. [5]. In some cases repeat studies are conducted varying the conditions of the assay, for example, three doses administered over a 72-h period (data not shown). An example of a repeat study that would be run using a 72-h regimen would be the testing of a genotoxin that needs bioactivation (such as cyclophosphamide and 4-nitroquinoline *N*-oxide), which may require more time to accumulate the reactive metabolite. Testing of different dose regimens is currently under investigation. For these studies, models received on Tuesday are placed into fresh medium and allowed to incubate for 1 h followed by replacement of the media with fresh medium containing cytoB and dosing of the test compound. Subsequent dosing and harvesting are the same as described above.

2.2. Key aspects that can impact the assay

2.2.1. Shipping issues

The RSMN assay in EpiDerm™ has been successfully transferred to the European-based laboratories, Henkel AG & Co. KGaA, Duesseldorf, and L'Oréal Life Sciences Research, Paris, France [8]. One of the key aspects is the overnight shipment of models maintained in a stable "cool" environment, especially for European laboratories. Because the models are constructed with primary proliferating cells, storage conditions during transport could affect the quality of the models and the consistency of results. A delay of one day may compromise the models and also leads to the inability to complete the assay during a normal working-week.

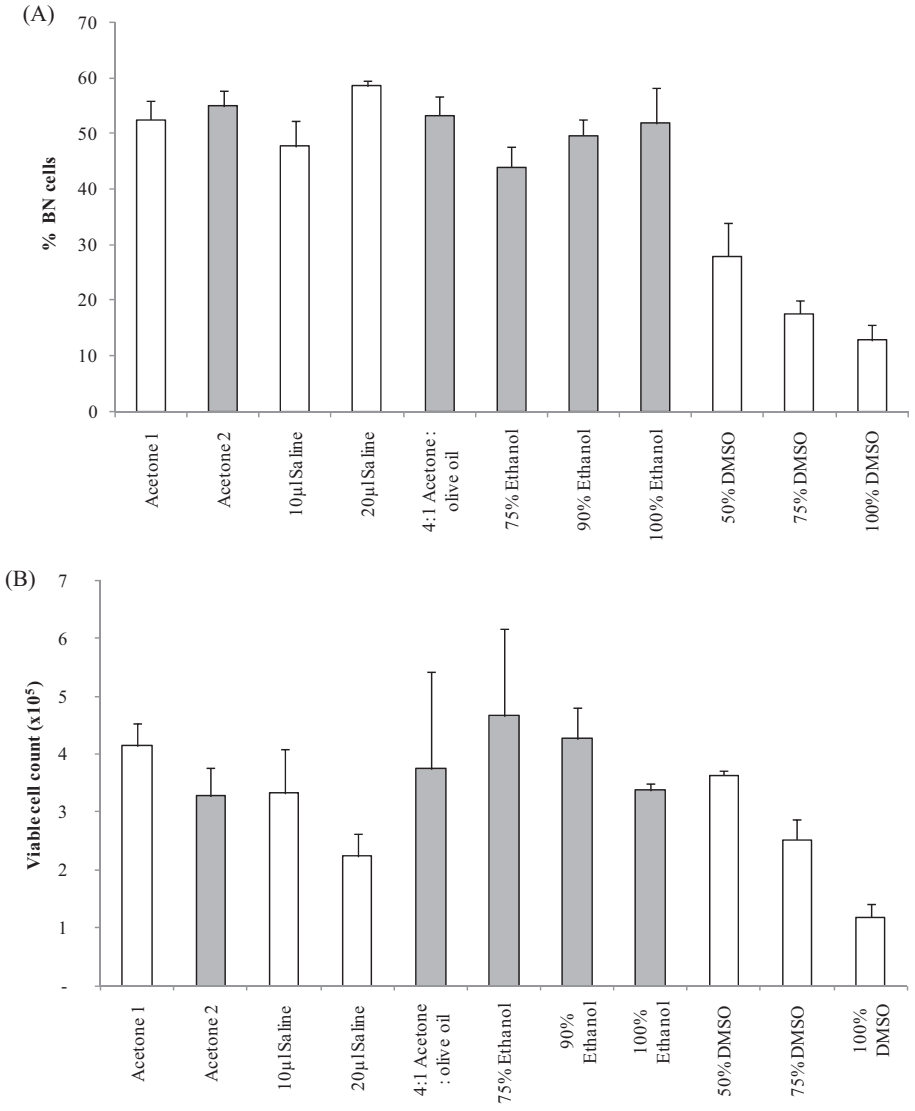

Fig. 2. Effect of different solvents on (A) percentage of bi-nucleated cells and (B) viable cell recovery of models treated with different solvents. Open bars are results from experiment 1 and closed bars are from experiment 2. Values are mean of 3 different EpiDerm™ models ± SD.

2.2.2. Solvents

Most studies conducted to date in the RSMN assay have utilized acetone as the solvent since this is a common dosing vehicle for rodent carcinogenicity studies with dermal exposure. To broaden the application of the assay, it was important to define the range of solvents that are compatible with the system. Therefore, EpiDerm™ models were treated with saline, ethanol, acetone, acetone/olive oil, or DMSO at different concentrations and in different dosing volumes. The percentage of bi-nucleated cells, as well as the viable cell counts was compared (Fig. 2). DMSO was the only solvent that affected the percentage of bi-nucleated cells (Fig. 2A). A large decrease in the percentage of binucleated cells was observed at 50%, 75% and 100% DMSO, with only 13% binucleated cells present after exposure to 100% DMSO. The decrease in the percentage of bi-nucleated cells at 75% and 100% DMSO was concomitant with a decrease in the number of viable cells recovered (Fig. 2B). Treatment with 20 μL saline decreased the viable cell recovery (2.2×10^5 cells per model) but not the percentage of bi-nucleated cells relative to acetone solvent (Fig. 2A). This is thought to be a consequence of interference of the air-liquid interface of the EpiDerm™ model, as has been observed previously [7]. Based on these results, DMSO and 20 μL saline are not considered appropriate solvents for the RSMN in EpiDerm™ assay, whereas 10 μL of acetone, saline, 4:1 acetone/olive oil, and ethanol are acceptable.

2.2.3. Training of scorers

During the transfer of the RSMN assay to additional laboratories, some variations in the scoring of micronuclei were noted, with some laboratories scoring higher frequencies of micronuclei than others using the same set of slides. To standardize the scoring of slides, all participating laboratories met to come to a consensus on how to score cells for both the percentage of bi-nucleation and for the presence of micronuclei. An atlas was generated, using images collected from all participating laboratories, showing cells that contain scorable micronuclei and examples of cells containing artefacts that might be mistaken for micronuclei. The harmonized scoring atlas is illustrated in Figs. 3–6 and is discussed in Section 4.

3. Detailed protocol for the RSMN assay

3.1. Experimental design and methodology

Each study consists of a dose range-finding assay and at least one definitive assay to determine the genotoxic potential of the test article. 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 assay. At this early stage of the assay, at least two valid studies, based on at least duplicate EpiDerm™ models for each dose of control and test article, are recommended. With further experience, repeat assays for clearly positive or negative results may not be needed. The genotoxicity of the test article is evaluated on the basis of the statistical significance of the micronucleus frequency in models with a percentage survival of at least 40% (based on the percentage of bi-nucleation or live cell count relative to vehicle-treated controls).

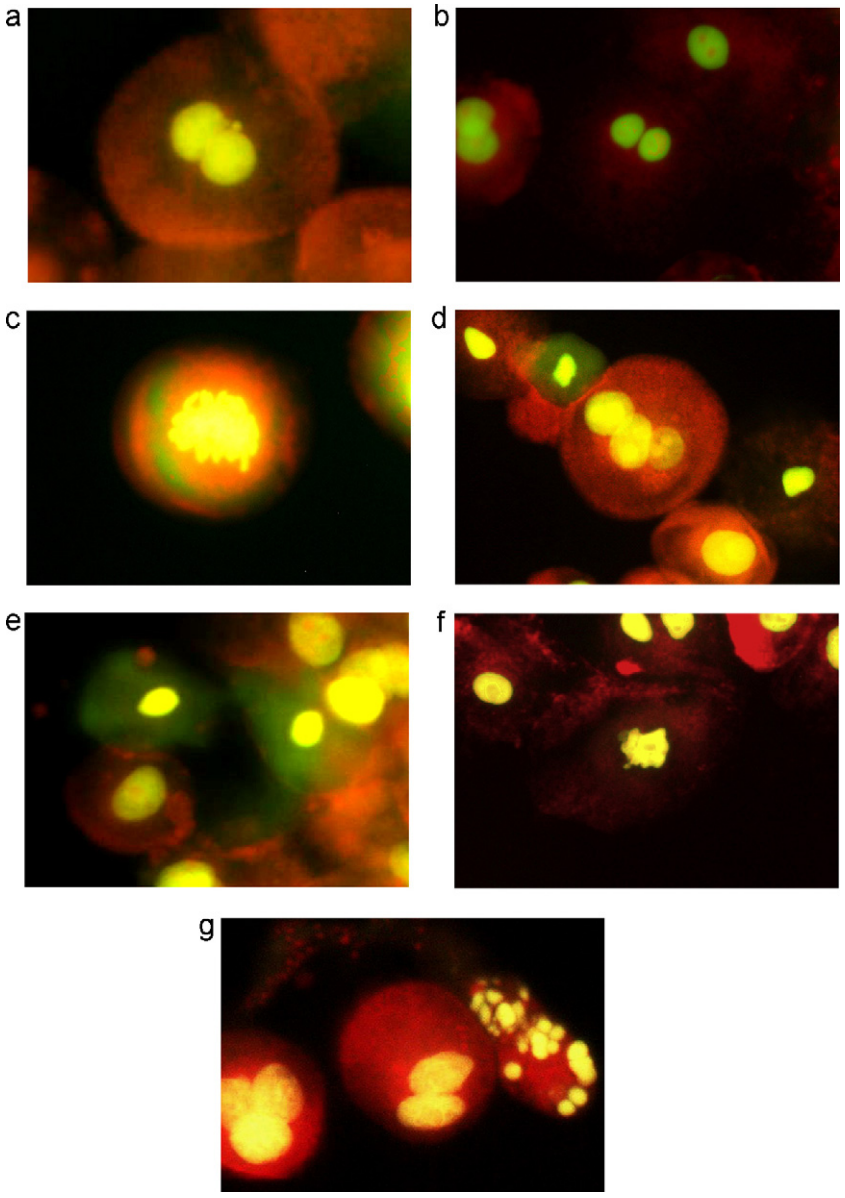

Fig. 3. Images to aid in scoring for percentage bi-nucleation. (A) Bi-nucleated cell with an intact cell membrane, which would be scored. (B) Bi-nucleated cell with a disrupted cell membrane should not be scored. (C) Mitotic cell, which should not be scored. (D) Tri-nucleated cell, which should not be scored. (E) Green cells are likely highly differentiated keratinocytes from the upper layers of the construct rather than the rapidly dividing basal cells. These should not be scored. (F) Cell with a misshapen nucleus, may be beginning of apoptosis. Only cells with round nuclei should be scored. (G) The cell on the right is likely apoptotic, note the degrading nucleus.

3.2. Standard chemicals, reagents and medium

Trypsin (0.25%)–EDTA (0.02%) is from JRH Biosciences or equivalent sources. Trypsin neutralizing solution (Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM L-glutamine) and EDTA 1 g/L are from Quality Biological, or equivalent sources. Acridine-orange solution (10 mg/mL) and cytoB are from Sigma–Aldrich. All other standard reagents are from Sigma–Aldrich or Quality Biological. New Maintenance Medium (NMM) and Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-DPBS) are from MatTek.

3.3. Preparation of stock chemical solutions

3.3.1. Positive control – mitomycin C (MMC)

In order to promote consistent doses among experiments, a stock solution of MMC is prepared and used to make fresh dosing solutions for each day of an assay. Sterile tissue culture-grade water at room temperature (4.0 mL) is added to a vial of MMC (2 mg per vial) to create a stock of 0.5 mg/mL. The vial is then vortexed until the MMC is completely dissolved and no visual evidence of purple precipitate is evident. A 100-μL aliquot of MMC stock (0.5 mg/mL) is transferred into cryovials for storage at −15 to −25 °C for up to one year.

On each day of dosing, a frozen vial of MMC stock is thawed at room temperature and examined for precipitate. Full solubilisation of the MMC is critical to reduce assay variability. If a precipitate is observed, the vial should be vortexed and/or sonicated to achieve a uniform suspension. If precipitate remains, the aliquot should be discarded. Appropriate dosing solution(s) are prepared in acetone (generally 3 μg/mL; see examples in Table 1). The prepared dosing solution(s) are discarded after use so that each frozen stock is used only once.

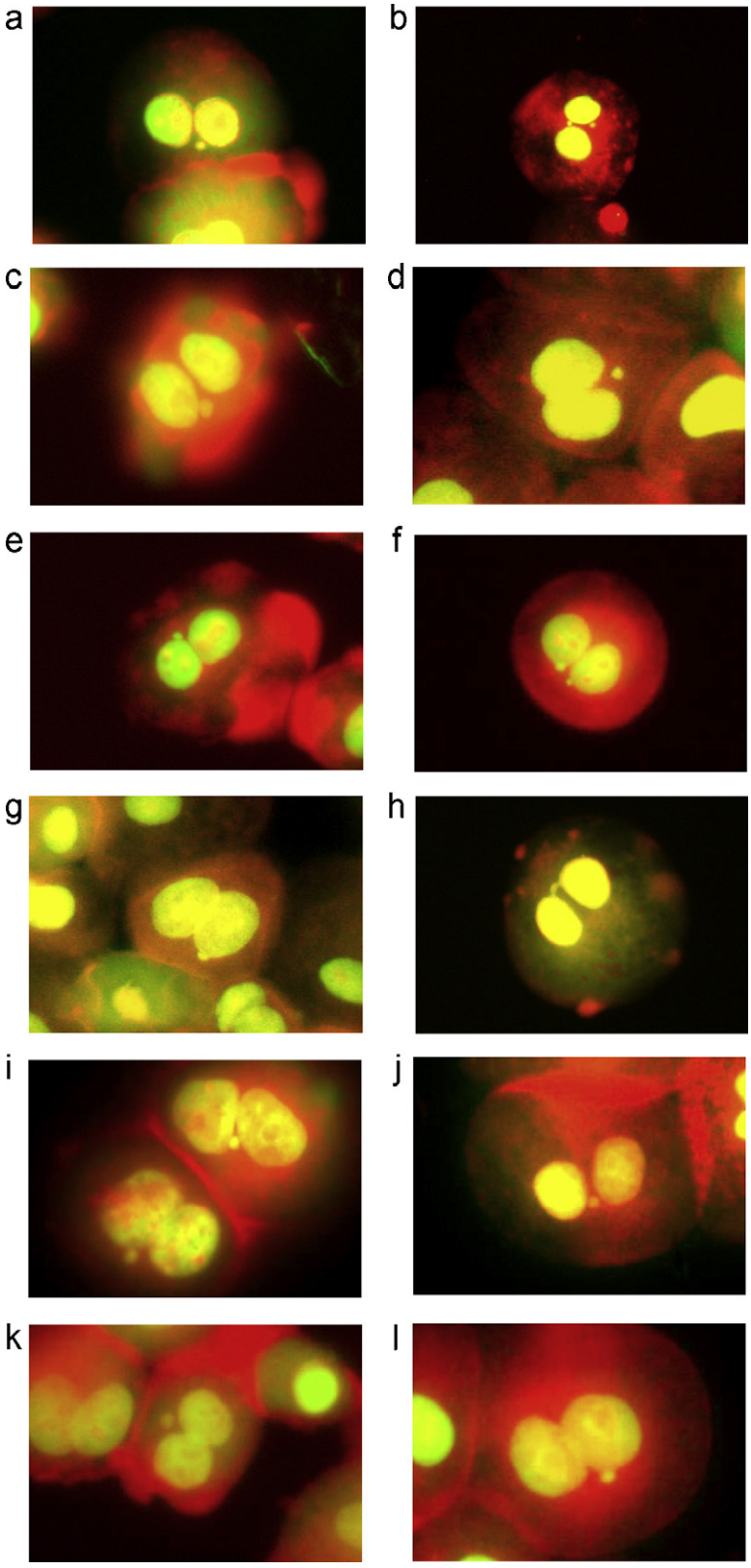

Fig. 4. Images of binucleated cells positive for micronuclei. (A) Typical micronucleus is round, found near the main nuclei, and matches the colour and intensity of the main nuclei. (B) Binucleated cells containing two micronuclei are counted as a single micronucleated cell. (C) Micronucleus touching the main nucleus. (D) Micronucleus is well separated from the main nuclei. (E) Micronucleus is overlapping one of the main nuclei but is still discernable as having a separate boundary. (F) Two micronuclei, each overlapping one of the main nuclei. Boundaries can be identified by changing the plane of focus. (G) Micronucleus is overlapping, but with careful focusing a clear boundary can be observed. (H) Nucleocytoplasmic bridges are rarely observed in this assay but should be noted. (I) Two binucleated cells containing a micronucleus. Note that each micronucleus matches the colour and intensity of the main nuclei in the same cell, even though they do not necessarily match each other. (J) The micronucleus only needs to match one of the main nuclei if the main nuclei do not perfectly match each other. (K) Micronucleus is clearly separated from main nuclei. (L) Micronucleus is touching the main nucleus.

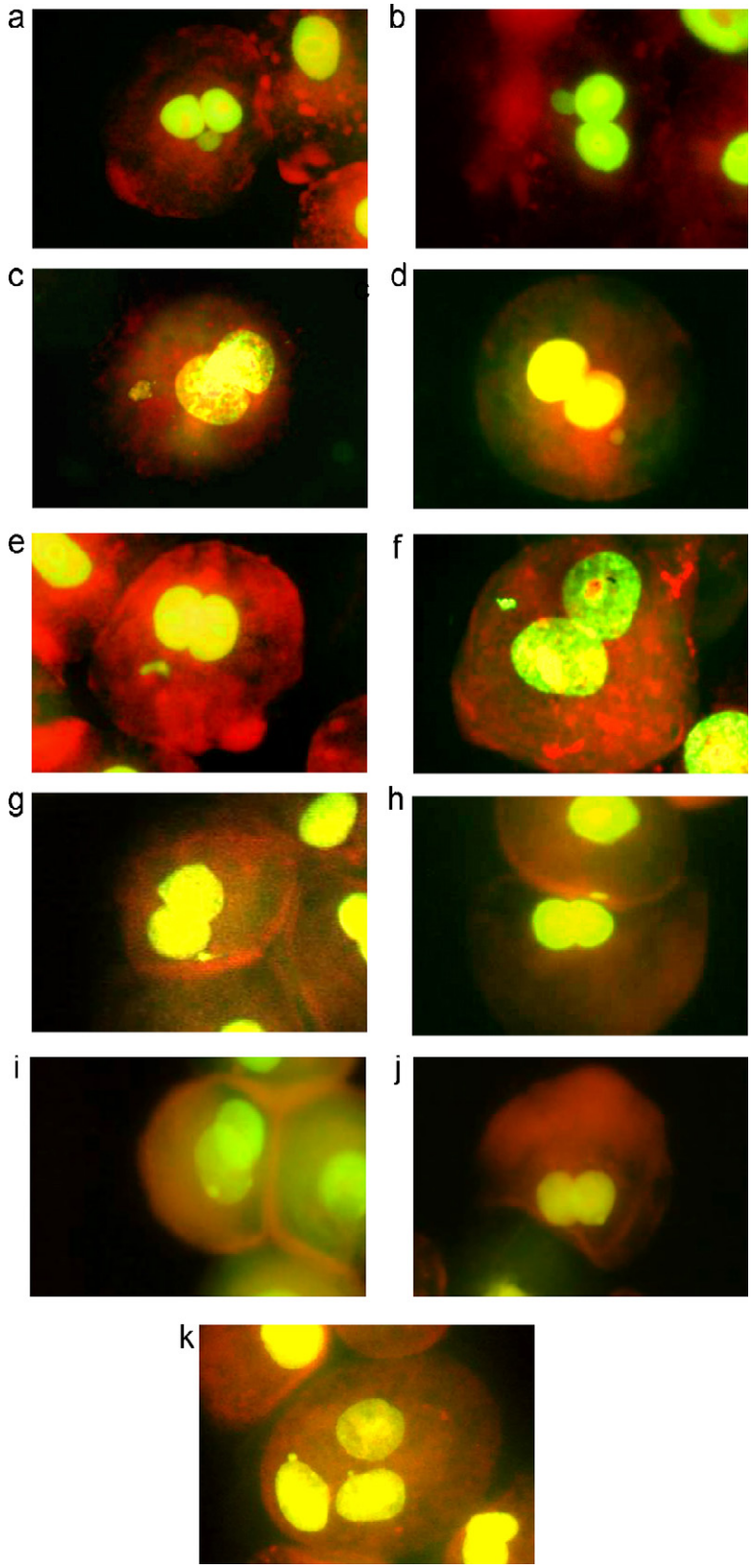

Fig. 5. Examples of bi-nucleated cells that would not be scored as positive for micronuclei. (A) The smaller nuclear object exceeds the maximum size of 1/3 diameter of the main nuclei to be considered a true micronucleus. (B) This object is too large and does not match the intensity of the main nuclei. This may be a nuclear extrusion. (C) This object is too close to the edge of the cell and is does not match the intensity of the main nuclei. (D) Object is the right size and shape but does not match the intensity of the main nuclei. (E–G) Examples of objects that do not have the smooth, round shape of true micronuclei. These are likely to be dust particles. (H) This object is not round, and is also located on the periphery of the neighbouring mononucleated cell rather than near the main nuclei of the binucleated cell. (I and J) These objects look like micronuclei but are completely within the boundary of the main nucleus so they are not counted. (K) Micronuclei in a tri-nucleated cell are not scored.

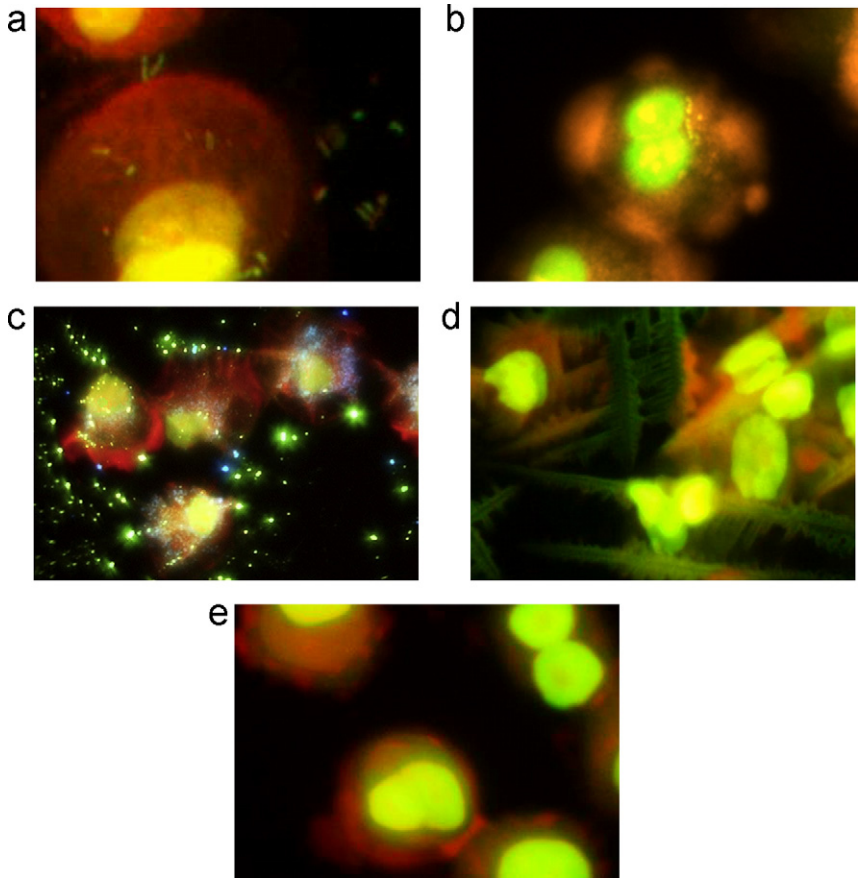

Fig. 6. Examples of technical difficulties that can hinder scoring. (A) Bacterial contamination. Bacterial DNA observed outside of the cell can help identify this as contamination. (B) Bacterial contamination appearing as small positive staining particles near the main nucleus. (C) Benzo(*a*)pyrene precipitate, which fluoresces with the same excitation and emission spectra as acridine orange stained DNA. (D) Crystallization during the fixation process can obscure scoring. (E) Cells that do not spread out well following KCl treatment have a reduced cytoplasmic volume, which can make identifying bi-nucleated cells and micronuclei more difficult.

Table 1
An example preparation of MMC in acetone.

Dose level	Volume of stock/diluted stock added (mL)	Volume of acetone added (mL)	Final concentration (μg/mL)
Dose 1	0.1 (0.5 mg/mL stock)	4.9	10
Dose 2	1.5 (10 μg/mL diluted)	3.5	3
Dose 3	0.5 (10 μg/mL diluted)	4.5	1

3.3.2. *Cytochalasin B (cytoB)*

A 3 mg/mL stock solution of cytoB is prepared by adding 3.3 mL DMSO to a 10 mg vial of cytoB (or 0.33 mL to a 1 mg vial). DMSO is used to prepare the cytoB stock-solution to ensure chemical stability of the solution according to the information provided by the manufacturer. The vial is vortexed until the cytoB is completely dissolved. Aliquots of cytoB (3 mg/mL) are transferred into cryovials for storage at −15 to −25 °C for up to one year.

On each day of dosing, an aliquot of cytoB stock-solution is thawed and diluted in NMM to the required concentration (generally 3 μg/mL). Each frozen aliquot is only used once. NMM supplemented with cytoB is warmed to 37 °C before use. Any unused NMM with cytoB is discarded.

3.3.3. *Acridine-orange solution*

A 40 μg/mL staining solution of acridine orange is prepared by adding 1.0 mL of a 10 mg/mL acridine-orange solution (solution purchased from the manufacturer is preferred to the powder form) to 249 mL Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-DPBS). The vial is vortexed to mix evenly. The final stain solution is stored at 2–8 °C and protected from light. It is stable for up to four weeks.

3.3.4. *Test-article preparation*

The test article is freshly prepared on each day of dosing. A volume of 10 μL of each test-article dilution is applied to the upper surface of the EpiDerm™ model. The preferred solvent for the test chemicals is acetone. Solvents such as ethanol and water may be used as an alternative (as discussed in Section 2.2.2). Although there is no maximum concentration recommended by the OECD [11], the COLIPA Task Force decided that the highest concentration in the range-finding assay should be based on the solubility of the test article in the chosen solvent, up to a maximum concentration of 10% (w/v) (i.e. 100 mg/mL).

3.3.5. *Controls*

Each assay includes a solvent control and a positive control. A volume of 10 μL solvent (typically acetone) is used as the solvent control. The positive control, 10 μL of 3 μg/mL MMC (in acetone) is tested in each assay.

3.4. *Receipt of EpiDerm™*

The EpiDerm™ Skin Model, EPI-200-MNA-kit includes the New Maintenance Medium (NMM, containing keratinocyte growth factor) and Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline

(CMF-DPBS). The Standard EpiDerm™ kit (EPI-200) includes a small amount of medium that is not compatible with the RSMN assay. It is crucial to use the New Maintenance Medium to maintain proliferation of the cells in the micronucleus assay. Additional NMM can be ordered separately from MatTek as needed. EPI-200-MNA-D2-254 (donor 254) was used in the COLIPA project but the RSMN assay has been shown to work with other EpiDerm™ donors from MatTek [7]. Upon receipt of the EpiDerm™ kit (EPI-200-MNA), the solutions are stored as indicated by the manufacturer. Each kit contains 24 EpiDerm™ Skin Models in a 24-well tray, which are stored at 2–8 °C until used.

On the day of receipt, an appropriate volume of NMM is warmed to approximately 37 °C. Aliquots of 1 mL of assay medium are transferred into the appropriate wells of 6-well plates. Each EpiDerm™ model is inspected for air bubbles between the agarose gel and the Millicell® insert prior to opening the sealed package by looking at the models from the underside of the sealed plate. Models with air bubbles greater than 50% of the Millicell® area or models with defects such as blisters that cover greater than 50% of the model (which indicates significant model detachment) are not used. It is also important to check whether there is any liquid on the model surface; if there is, it should be very carefully removed with a sterile cotton swab. The 24-well shipping containers are removed from the plastic bag and an appropriate number of EpiDerm™ models are transferred aseptically into the 6-well plates. The EpiDerm™ models are incubated at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% CO_2 in air (standard culture conditions) overnight. If the 72-h protocol is used, the models are incubated for 1 h before dosing to allow completion of the assay during a normal working-week.

3.5. Assay procedure

An overview of the RSMN-assay timeline is illustrated in Fig. 1.

3.5.1. Initial dosing

The models are re-fed with fresh, warm NMM containing 3 μg/mL cytoB. Upon re-feeding, the models are dosed with 10 μL of the test article, vehicle control, or positive control compounds. Since acetone (a volatile solvent with low viscosity) is a common solvent in the RSMN assay, care must be taken that the volumes pipetted are accurate. The dosing solution is placed on the surface of the models, tilting the plate to ensure that the surface of the model is covered by the dosing solution. The models are incubated under standard culture conditions.

3.5.2. Second dosing

After 24 ± 3 h, the models are re-fed with fresh warm NMM containing 3 μg/mL cytoB, dosed again, and incubated under standard culture conditions. The dosing solutions and the 3 μg/mL cytoB in NMM are prepared fresh on the day of use.

3.5.3. Third dosing

For chemicals that require metabolic activation, initial results suggest that a three-dose protocol over 72 h may improve detection (data not shown). Even though there is insufficient data to recommend routinely including a third dosing for an unknown test article, we recommend considering a repeat trial that includes a 72-h exposure if initial trials are negative or equivocal for an unknown chemical. If the protocol is modified to include a 72-h exposure, the models are incubated for only 1 h in fresh medium on the day of arrival, then re-fed with fresh warm NMM containing 3 μg/mL cytoB, and dosed as described above. The models are incubated under standard culture conditions. Twenty-four (±3) hours later, the models are re-fed with fresh warm NMM containing 3 μg/mL cytoB, the second dose of the test article is administered, and the models are incubated under standard culture conditions. The third dosing is administered 24 ± 3 h after the second dose and the models are incubated until cell harvest, 72 h after the first dosing.

3.5.4. Cell harvest

Forty-eight (±3) hours after initial treatment, the models are trypsinized to obtain a single-cell suspension of cells from the basal layers. To avoid over-trypsinization and to maintain consistency in the single-cell suspensions, we recommend trypsinizing models in groups of six models or fewer per technician, keeping the remaining models under standard culture conditions.

Each EpiDerm™ model insert is placed in a well of a 12-well plate containing 5 mL CMF-DPBS at room temperature for 5–15 min. Each insert is removed from its well, inverted to decant the CMF-DPBS, blotted on a paper towel, then placed in a new well containing 5 mL of EDTA (0.1%, 1 g/L) and incubated at room temperature for 15 min. Each model insert is then removed, inverted to decant excess EDTA, blotted, then placed in a new well containing 1 mL of warm (~37 °C) trypsin–EDTA solution. Warm trypsin (0.5 mL) is added inside each insert and the models are incubated for 10–15 min at 37 °C. After this incubation, each insert is held with forceps over a new well of a 12-well plate containing 1 mL of fresh warm trypsin–EDTA. The model is carefully separated from the supporting membrane by gently lifting the edge of the model with another pair of fine forceps. Both the detached model and the supporting membrane are transferred to the new well. The insert is thoroughly rinsed (4–6 times) using the trypsin–EDTA in the well to collect any remaining basal cells left on the supporting membrane into the well, after which the insert is discarded. The model is gently agitated to release additional attached cells from the detached model, which is now primarily *stratum corneum* and which is resistant to further trypsinization. At this point, the remaining tissue can be discarded [5]. Any cell clumps can be disrupted by repeatedly drawing the cell suspension in trypsin–EDTA into a pipette no larger than 2 mL and gently expelling the solution. The single-cell suspension (~1.5 mL) is transferred to a 15-mL conical tube containing 1.0 mL of warm DMEM with 10% FBS to inactivate the trypsin. No more than 5 min should elapse between detaching the model and the trypsin neutralization step to ensure optimal cell condition. Alternatively, trypsin can be neutralized by adding 1 mL of warm DMEM with 10% FBS directly into the well and the neutralized cell suspension can then be transferred to an empty, labelled 15-mL tube. A sample of cell suspension is diluted with Trypan blue solution and counted using a haemocytometer to obtain a cell count and determine the proportion of live cells of each treatment compared with the control. Other methods to obtain a live cell count, such as the Easy Count™ (Immunicon Huntingdon Valley, PA) can be used.

3.5.5. Fixation

The cell suspension is centrifuged (100 × *g* for 5 min) at room temperature, and the supernatant is carefully removed. The cell pellet is loosened by gentle flicking the base of the centrifuge tube and 1 mL of warm (~37 °C) KCl solution is added slowly down the side of the tube while gently shaking (~500 rpm if using a Vortex type mixer) the cell suspension. After approximately 3 min, 3 mL of fresh (prepared on the day of use), cold (4 °C) methanol/acetic acid (3:1) fixative is added slowly to the cells, and the cell suspension is centrifuged at 100 × *g* for 5 min. Each "slow" addition process should take approximately 10 s, and care should be taken that all cell suspensions receive identical treatments. Slides are prepared as described below. If significant salt crystallization occurs on the slide (Fig. 6D), a second fixation step can be used, which reduces salt crystallization but tends to reduce the cell yield. For the second fixation, 4 mL of cold fresh 40:1 or 99:1 methanol/acetic acid is added to the cell suspension after the first fixation and centrifu-

gation. After the second fixation, the cell suspension is centrifuged at 100 × g for 5 min and slides prepared as described below.

3.5.6. Slide preparation and acridine-orange staining

After centrifugation, all but a small portion (approximately 50–200 μL) of the supernatant is removed, and the cell pellet is loosened by gently flicking the centrifuge tube. A single drop (i.e. 15–20 μL) of the cell suspension is gently dropped from a pipette 2–5 cm above a clean, dry microscope slide (Gold Seal®, Beckton Dickinson & Co., Catalogue number 3050) that is either flat or slightly tilted. Two slides are prepared from each EpiDerm™ model, whenever possible.

After the slides are completely dry, they are immersed in acridine-orange solution for 2–3 min, immediately rinsed 3 times with CMF-DPBS (each rinse lasting at least 1 min), and allowed to dry. Used solution is discarded. Staining slides on the day of slide preparation can reduce the appearance of salt crystals, which can interfere with scoring (Fig. 6D). Stained slides are stored in the dark at 2–8 °C. Prior to analysis, a drop of CMF-DPBS is put onto the slide, a coverslip is added and the slides are examined by use of a fluorescence microscope with 40×- or 60×-objectives, equipped with a blue filter (e.g. Opelco).

4. Slide scoring

All slides should be blind-coded before scoring. The experiment may be qualified to determine if it is a valid assay before blind scoring commences by scoring a positive control slide and a negative control slide, to check for sufficient bi-nucleation (at least 25% binucleated cells in the negative control) and induction of micronuclei (significantly higher percentage of micronucleated bi-nucleated cells in the positive control than in the negative control). However, the slides used for qualifying the assay should then be blind-coded and re-scored with the rest of the experimental slides.

If the acridine-orange staining fades, slides that require analysis can be re-stained. For re-staining, slides should be immersed for 10–15 s in the acridine-orange staining solution, followed by washing as described above. The re-stained slides are evaluated to check for staining intensity, and if needed, re-stained for an additional 5–10 s, and washed again. This may be repeated several times until the staining is intense enough to score the slides.

4.1. Cytotoxicity

For an evaluation of cytotoxicity, at least 500 cells are scored per EpiDerm™ model to determine cell proliferation, as measured by the percentage of mononucleated, bi-nucleated and multinucleated cells. The analysis for toxicity is performed in an analysis separate from that of the micronucleated cells in order to avoid bias in cell selection for quantifying micronuclei. The cytoplasm should be stained red and should be relatively intact, as illustrated in Fig. 3A. Free nuclei or cells without a clear cell membrane (Fig. 3B) should not be included in the analysis. Cells with green staining cytoplasm, pictured in Fig. 3E, which are likely more highly differentiated [12] are not included in the analysis. The nuclei should be approximately equal in size, and can overlap, as they often do in the RSMN assay, particularly if the cells do not spread out well (Fig. 6E). Care must be taken to discern whether there is a nuclear boundary between them for classification as a bi-nucleated or multinucleated cell. Slides with fewer than 500 scorable cells are not evaluated for micronucleus induction. The percentage bi-nucleated cells in the individual untreated/solvent control models should be 25% or more. If the value in any individual model is lower than 25%, then there is likely a technical issue and the slides from that model should not be analyzed further.

The percentage survival for each EpiDerm™ model in each treatment condition is calculated as the ratio of the percentage of bi-nucleated cells in treated models compared to the average percentage of bi-nucleated cells in the solvent control models. Slides with fewer than 500 scorable cells are averaged into the percentage survival calculation as 0% survival (100% toxicity). The cytokinesis-block proliferation index (CPBI) or replicative index (RI) can also be used as described in the OECD guideline [11]. Another end point of cytotoxicity is the relative live cell count. This is calculated by comparing the yield of live cells (evaluated by Trypan-blue exclusion) from each chemically treated model to the average of the solvent control model. In the COLIPA project [5,6,8], the relative percentage of bi-nucleation was used as main cytotoxicity parameter, although the relative percentage of live cells was the more sensitive cytotoxicity parameter in other studies, particularly with liquid test articles [7]. In a typical assay, a series of concentrations of each test article is evaluated including concentrations that induced no toxicity up to concentrations that induce 55 ± 5% toxicity (45 ± 5% survival), as suggested in the draft OECD guidelines [11]. For non-toxic test articles, a top concentration based on the solubility of the test article in the vehicle, up to 10% (100 mg/mL) is recommended and was used in the RSMN Pre-validation Project [8].

4.2. Analysis of micronuclei in binucleated cells

Only EpiDerm™ models showing greater than or equal to 40% survival (based on relative percentage of binucleation, CPBI, RI, or relative live cell count) are scored for micronucleus frequency. One thousand binucleated cells with intact and red stained cytoplasm are scored per model (or at least 500) to determine the frequency of micronucleated cells in the bi-nucleated cell population. Models with fewer than 500 scorable cells will be recorded as “not scorable”. To avoid any bias in the selection of cells for analysis, the quantification of micronuclei is conducted separately from the analysis of cell proliferation (mononucleated, bi-nucleated, or multinucleated).

The criteria of Fenech et al. [13,14] are used to select binucleated cells for the analysis of micronuclei and for classification of micronuclei. Criteria for binucleated cells are: (a) the two nuclei should be approximately equal in size and staining, and (b) the binucleated cells should have intact cytoplasmic membranes (note: the cells can touch or overlap as long as the cytoplasmic boundary is distinguishable). Micronuclei observed in cells with more than two nuclei, or in cells with a single nucleus are not scored. Cells with green staining cytoplasm, or without a relatively intact cell membrane, are not analyzed.

Micronuclei are characterized by the following criteria (examples of cells positive for micronuclei are illustrated in Fig. 4):

a. They must be stained the same colour and intensity as the main nuclei. Lighter objects may be nuclear extrusions (Fig. 5B) or oil droplets (Fig. 5C and D).
b. Micronuclei are morphologically similar to the main nuclei but smaller. There is no lower limit on the size of micronuclei as long as the acceptable shape of the micronuclei can be verified. The diameter of the micronucleus must be less than 1/3 that of the main nuclei in a binucleated cell (or 1/9 of the area of a main nucleus). Examples of artefacts that are too big to be counted as micronuclei are illustrated in Fig. 5A and B.
c. Round or oval in shape. Misshapen micronuclei are likely to be dust (Fig. 5E–H).
d. No link to one of the main nuclei, though they may touch or overlap the main nuclei (see Fig. 4F and G). MN in the RSMN assay frequently touch or slightly overlap the edge of a main nucleus; they thus require careful examination focusing up and down to discern whether there is a nuclear boundary.

The number of micronuclei per cell is noted, but analysis is based on the number of micronucleated cells. Other effects such as nucleoplasmic bridges, apoptosis, necrosis, micronuclei in mononucleated cells, etc. can also be analyzed in this assay, but the assessment of genotoxicity to date has been based on induction of micronuclei in binucleated cells.

4.3. Scoring atlas

Figs. 3–6 contain examples of cell images depicting different types of cells and micronuclei obtained in the RSMN assay.

4.3.1. Examples of cells for analysis of proliferation

Fig. 3A shows a typical bi-nucleated cell with fully intact cytoplasm. Cells with incomplete cytoplasms can be analyzed for proliferation as long as the cell is distinct from adjacent cells and the nuclei are clearly associated with the cell. Fig. 3B illustrates a bi-nucleated cell with no intact cytoplasm that should not be scored. Cells with three or more nuclei are often observed and should be counted as multinucleated cells. Micronuclei that occur in multinucleated cells are not included in the micronucleus analysis. Bi-nucleated cells should be examined carefully for overlapping nuclei, to prevent misclassification. Mitotic cells (Fig. 3C) or cells with irregularly shaped nuclei (Fig. 3F) are not included in the analysis of mono-, bi- or multi-nucleated cells. Green, differentiated, cells (Fig. 3E) or apoptotic cells (Fig. 3G) are also excluded.

4.3.2. Typical examples of binucleated cells with micronuclei

Fig. 4A–D, J and K shows the examples of binucleated cells containing micronuclei that are separate from the main nucleus. Nuclei are of approximately equal size and intensity and the cells have clear cytoplasmic boundaries. Examples of binucleated cells with small touching micronuclei are shown in Fig. 4E–G, I and L. All of these micronuclei can be counted because they have distinct boundaries that can be observed when the microscope is focused carefully. It should be noted that two micronuclei in one cell are recorded, but analyzed as one micronucleated cell for statistical purposes. Fig. 4H shows a nucleoplasmic bridge, which has been rarely observed in this assay.

4.3.3. Examples of artefacts that may be misidentified as micronuclei

The scoring of the slides can be affected by a number of artefacts, illustrated in Fig. 5, which may hinder scoring unless they are correctly identified. Fig. 5A and B shows the examples of artefacts that are too large to score as micronuclei. Fig. 5C and D shows the artefacts that are the correct size as a true micronucleus, but do not match the intensity of the main nucleus. Fig. 5E–H shows the examples of artefacts that are not round, as required for true micronuclei. Dust particles can often be confused with micronuclei, but are usually not smooth and round-shaped. Similar particles appearing outside the cells can help identify dust particles inside the cells that might be mistaken for micronuclei. Fig. 5I and J shows the objects that may be micronuclei but are not counted because they are completely within the boundaries of the main nucleus, rather than simply overlapping as shown in Fig. 4E–G, I and L. Fig. 5K illustrates micronuclei in a tri-nucleated cell, which would not be counted. Only micronuclei in binucleated cells are included in the analysis.

4.3.4. Technical difficulties

Technical difficulties that may impact scoring are depicted in Fig. 6. Fig. 6A and B illustrates bacterial contamination, which can sometimes interfere with scoring. Fig. 6C illustrates the problem of precipitation of high concentrations of test articles. Chemicals that fluoresce at the same excitation and emission wavelengths as acridine orange appear to be the same colour as nuclei and micronuclei. An example of this is shown in Fig. 6C for benzo(*a*)pyrene. A common problem of the slide fixation is the precipitation of crystals, shown in Fig. 6D. The formation of crystals can be decreased by washing the slides for a second time (see Section 3.5.4). If the crystals do not obscure the cells too much to observe micronuclei, the slides can still be included in the scoring. Some single-cell suspensions do not spread out well, and can result in a reduced cell volume that makes it more likely that nuclei will overlap, as illustrated in Fig. 6E. These slides can still be scored if enough micronuclei are observable in the positive control slides.

4.4. Statistical analysis

Statistical analyses are normally based on an experimental unit that is the largest entity on which treatments are separately applied. For the RSMN assay, that would be the individual EpiDerm™ models rather than the cells isolated from the models, even though the MN response is measured at the cell level. An analysis of historical control data from a variety of laboratories performing the RSMN assay indicates there is little model-to-model variability in these data so that the cell (as opposed to the model) can be treated as the effective statistical unit. The data follow a binomial distribution. An advantage of this is the fact that this makes the analysis most sensitive because it maximizes the effective sample size (1000 cells/model/treatment group compared to just 2–3 models/treatment group) in the statistical analysis. In the RSMN assay, this means that the cell is preferable to the model as the unit of statistical analysis and is consistent with the current standard *in vitro* MN assays. Another advantage of this choice is that binomial test methods (Fisher exact and Cochran–Armitage trend tests) can be used and will be more sensitive to treatment effects. Based on this, a one-sided Fisher's exact test is used to determine the statistical significance of differences between solvent control and each of the test-article treatments, where $p < 0.05$ will be considered a significant positive response. A Cochran–Armitage test $p < 0.05$ is used to evaluate dose response.

4.5. Criteria for the determination of a valid test

The criteria for the determination of a valid test were according to Aardema et al. [8], in which the method was being harmonized between laboratories. These criteria are based on previous studies and the current guideline:

1. The positive control compound causes a statistically significant increase in the frequency of micronucleated bi-nucleated cells.
2. At least 3 concentrations of the test article meet the acceptance criteria below.
3. At least 2 models per treatment passing the following criteria:
   (i) At least 50,000 viable cells per untreated/solvent control model.
   (ii) The average percent bi-nucleation of the untreated/solvent control is at least 25%.
   (iii) The percentage of micronucleated bi-nucleated cells in the solvent control treated models is within the acceptable historical range for the laboratory. An average of around 0.08% (and a range of 0–0.5%) has been obtained across a number of laboratories [7].
   (iv) The number of models should be 3 per treatment with at least 2 models passing all criteria.
   (v) At least 500 binucleated cells that can be evaluated for micronuclei [6].

4.6. Criteria for judging a positive or negative assay

The criteria below are used to judge the outcome of an assay and are the same as those previously used [8], with the exception of the use of the Cochran–Armitage trend test, which we have recently included as an additional method (see point 4):

1. For each assay, any statistically significant increased data point (Fisher's exact test $p < 0.05$) relative to controls is flagged.
2. An experiment is considered positive for genotoxicity if it has one or more concentrations that produce a statistically significant increase in the percentage of micronucleated cells. The evaluation should consider biological relevance, i.e. dose-response (trend test, see 4 below) and the lab's historical control range.
3. A chemical is called positive for genotoxicity overall if it has at least 1 experiment with 2 or more concentrations producing statistically significant increases in micronucleus induction, or one concentration that produces a statistically significant increase in micronucleus induction that is reproducible in 2 independent studies. The evaluation should consider biological relevance (trend and the historical control range).
4. The results of the Cochran–Armitage trend test are used in the overall judgment of the response.
5. A study is considered negative if the above criteria are not met at doses below the $55 \pm 5\%$ toxicity limit ($45 \pm 5\%$ survival) or the highest tested concentration [11].

Further experience and a larger database for the RSMN assay may lead to modification of these assessment criteria and/or study design. For instance, greater power of the assay would be achieved by evaluating more cells, or by using a criterion that considered a chemical positive if it induced a statistically significant increase in 2 or more concentrations or in 1 concentration with a significant trend test in a single test. This would eliminate the need for a repeat study.

5. Conclusion

This paper describes the detailed methods and scoring criteria for the successful conduct of the RSMN using EpiDerm™ models. These methods have resulted in the successful transfer of the assay to a number of US and European laboratories as part of a COLIPA-sponsored project [8]. We hope that new laboratories conducting the RSMN in the future will find these resources valuable in helping to generate quality data. It is hoped that other laboratories will adopt the RSMN assay, which will then add to our understanding of the predictability of this promising new *in vitro* genotoxicity assay.

Conflict of interest statement

Erica L. Dahl, Rodger Curren and Greg Mun state that their laboratory may offer this assay on a fee-for-service basis in the future. Marilyn Aardema is now an employee of Bioreliance Corp. which will offer the RSMN assay commercially in the future. The other authors have no conflict of interest.

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