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International prevalidation studies of the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay: Transferability and reproducibility

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ABSTRACT

Recently, a novel *in vitro* reconstructed skin micronucleus (RSMN) assay incorporating the EpiDerm™ 3D human skin model (Curren et al., *Mutat. Res.* 607 (2006) 192–204; Mun et al., *Mutat. Res.* 673 (2009) 92–99) has been shown to produce comparable data when utilized in three different laboratories in the United States (Hu et al., *Mutat. Res.* 673 (2009) 100–108). As part of a project sponsored by the European cosmetics companies trade association (COLIPA), with a contribution from the European Center for the Validation of Alternative Methods (ECVAM), international prevalidation studies of the RSMN assay have been initiated. The assay was transferred and optimized in two laboratories in Europe, where dose-dependent, reproducibly positive results for mitomycin C and vinblastine sulfate were obtained. Further intra- and inter-laboratory reproducibility of the RSMN assay was established by testing three coded chemicals, *N*-ethyl-*N*-nitrosourea, cyclohexanone, and mitomycin C. All chemicals were correctly identified by all laboratories as either positive or negative. These results support the international inter-laboratory and inter-experimental reproducibility of the assay and reinforce the conclusion that the RSMN assay in the EpiDerm™ 3D human skin model is a valuable *in vitro* method for assessment of genotoxicity of dermally applied chemicals.

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1. Introduction

In vivo genotoxicity testing in experimental animals of cosmetics ingredients for products marketed in Europe was banned in March 2009 (EU 7th Amendment to the Cosmetics Directive [1]). Consequently, studies such as the *in vivo* bone-marrow micronucleus (MN) assay and other *in vivo* genotoxicity assays can no longer be used as follow-up assays to evaluate the genotoxic potential of chemicals that are positive in *in vitro* assays. *In vivo* studies are also impractical for large-scale chemical evaluation programs such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) [2], leading to an emphasis on the

use of *in vitro* assays. Although many *in vitro* genotoxicity assays have been routinely carried out for decades, the standard battery of tests has been shown to produce high rates of positive results that are often not confirmed in *in vivo* genotoxicity and/or rodent carcinogenicity tests, i.e. in some cases up to 95% “false” or “misleading” positive results [3,4]. False positive results in the standard genotoxicity assays are recognized as a critical issue in the genetic toxicology community worldwide and a number of multi-stakeholder groups are working to define improved approaches for assessing genotoxicity (e.g., International Life Sciences Institute-Human and Environmental Sciences Institute committee on The Relevance and Follow-up of Positive Results in *In Vitro* Genotoxicity Testing (IVGT); www.hesiglobal.org). For those situations where *in vivo* follow-up assays are not allowed (cosmetic ingredients in Europe) or not practical (e.g., REACH), this is a critical problem since reliance on *in vitro* assays solely to assess genotoxicity would result in many promising and safe ingredients not being developed.

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Table 1
Participants in the COLIPA 3D reconstructed human skin genotoxicity assay project (2007–2009).

Steering Committee			
Chair	Marilyn Aardema	The Procter & Gamble Company	Cincinnati Ohio, USA
Co-chair	Kerstin Reisinger	Henkel AG & Co KGaA	Duesseldorf, Germany
Members	Rodger Curren	Institute for InVitro Sciences	Gaithersburg, MD, USA
	Stefan Pfuhrer	The Procter & Gamble Company	Cincinnati Ohio, USA
	Cyrille Krul	TNO – Quality of Life	Zeist, The Netherlands
	Gladys Ouédraogo-Arras	L'Oreal Life Sciences Research	Aulnay sous Bois, France
COLIPA Project Management Representative from ECVAM	Monique Fairley	COLIPA	Brussels, Belgium
	Raffaella Corvi	European Center for the Validation of Alternative Methods	Ispra, Italy
Chemical Selection Committee			
Chemical Selection Expert team	David Kirkland	Covance Laboratories	Harrogate, UK
	Thomas Slaga	UTHSCSA-Pharmacology	San Antonio, TX, USA
	Johannes Doehmer	GenPharmTox BioTech AG	Planegg/Martinsried, Germany
	Günter Speit	Universität Ulm	Ulm, Germany
Laboratories involved in the RSMN project			
The Procter & Gamble Company	Marilyn Aardema, Brenda Barnett, Zubin Khambatta		USA
IIVS	Rodger Curren, Greg Mun, Erica Dahl		USA
L'Oréal	Gladys Ouédraogo, Brigitte Faquet, Anne-Claire Ginestet		France
Henkel	Kerstin Reisinger		Germany

Since skin is the first site of contact with maximum exposure to many different products, including cosmetics, assays using 3D human reconstructed skin (RS) models were chosen since they offer the potential for a more physiologically relevant approach to test dermally applied compounds. Although more detailed studies of RS models are needed, it is likely that they have more normal DNA repair and cell-cycle control since they are prepared from primary human cells and exist in a three-dimensional form, than cell lines used in standard genotoxicity assays. These models are also expected to provide a human metabolic capability that is more relevant than the exogenous rodent metabolizing enzymes currently used in standard *in vitro* genotoxicity assays. Since these skin models have a functional stratum corneum, they also provide a more relevant target-cell exposure, avoiding the non-physiological concentrations of chemicals and drugs that often occur in the current *in vitro* genotoxicity tests. It is anticipated that these features of the RS models could improve the predictive value of a genotoxicity assessment compared with that of existing *in vitro* assays.

We have previously reported the development and evaluation of intra- and inter-laboratory reproducibility of the RSMN assay using EpiDerm™ with a variety of chemicals across three laboratories in the United States [5–7]. The chemicals evaluated included model genotoxins exhibiting a range of mechanisms, e.g., cross-linking (mitomycin C [MMC]), alkylation (methyl methane-sulfonate [MMS]) and aneuploidy (vinblastine sulfate [VB]), establishing the ability of this model to respond to different types of damage. In addition, all three laboratories identified four dermal non-carcinogens, previously reported to be genotoxic in standard

assays, to be negative, showing that this assay has promise as a more predictive assay than standard *in vitro* genotoxicity assays. There was good reproducibility among different models containing keratinocytes isolated from human foreskin from four different donors. This is important since these primary cells have a finite lifespan and must be replaced by cells from a new donor when batches of cells are depleted. Initial results with chemicals that require metabolism are promising [8]. *N*-acetylation of aromatic amines has been demonstrated in the RS models [9], and recent results have shown the expression of xenobiotic metabolism genes to be similar to that found in normal human skin [10].

To extend these results, a global, multi-laboratory validation project for the RSMN assay was initiated in April 2007 and funded by COLIPA and the European Center for Validation of Alternative Methods (ECVAM). In this project RS models are also used to look at primary DNA damage with the Comet assay; Flamand et al. [11] had shown earlier that the UV-induced effects of two photogenotoxic compounds could be measured in RS models. The members of the Steering Committee for the project are listed in Table 1. Two independent (non-cosmetic company) laboratories, the Institute for In Vitro Sciences (IIVS) in the United States, and TNO in the Netherlands are involved in this project. This project follows a modular validation approach [12] and is divided into three phases: Phase 1 – transferability, optimization, and within-laboratory reproducibility with two model genotoxins; Phase 2 – between-laboratory reproducibility with three coded chemicals; and Phase 3 – increasing the domain of chemicals tested for predictive capacity and further evaluation of reproducibility. The chemicals for this project

Table 2
List of compounds tested.

Compound	CAS number	Carcinogenicity and genotoxicity data	Vehicle in RSMN assay	RSMN assay result
Mitomycin C	50-07-7	Carcinogenic in rats and mice (reviewed in Kirkland et al. [3]) Positive in <i>in vitro</i> genotoxicity assays and <i>in vivo</i> genotoxicity assays (reviewed in Kirkland et al. [3])	Acetone	+
Vinblastine sulfate	143-67-9	Carcinogenic in rats and mice [16] Positive in MLA [17], Ames and MNT [18] Positive MN and CA in mice <i>in vivo</i> [19]	Ethanol	+
<i>N</i> -Ethyl- <i>N</i> -nitrosourea	759-73-9	Carcinogenic in rats, positive in <i>in vitro</i> and <i>in vivo</i> genotoxicity assays (reviewed in Kirkland et al. [3])	Acetone	+
Cyclohexanone	108-94-1	A non-carcinogen [20,21] Non-genotoxic in <i>in vitro</i> assays (reviewed in Kirkland et al. [3])	Acetone	–

Table 3

Summary of the % BN cells and frequency of MN in acetone and ethanol control-treated tissues in two different laboratories.

	Vehicle control BN level (%)				Vehicle control MN level (%)			
	Acetone		Ethanol		Acetone		Ethanol	
	Henkel	L'Oréal	Henkel	L'Oréal	Henkel	L'Oréal	Henkel	L'Oréal
No. of tissues	12	9	12	6	12	9	12	6
Mean \pm SD	47.7 \pm 8.9	51.2 \pm 5.6	41.2 \pm 3.6	51.9 \pm 3.1	0.21 \pm 0.16	0.0 \pm 0.0	0.21 \pm 0.15	0.05 \pm 0.05
Maximum	63.5	58.8	47.8	56.8	0.60	0	0.60	0.10
Minimum	34.0	42.0	34.6	47.2	0	0	0	0

were identified by an independent Chemical Selection Expert team (Table 1).

This report describes the results of Phase-1 and Phase-2 studies for the RSMN assay using EpiDermTM. The laboratories involved in the RSMN project are shown in Table 1. Since the EpiDermTM models are manufactured in the United States, it was important to establish whether these could be shipped to Europe and used successfully in the RSMN assay. In Phase 1, the assay was transferred to two European laboratories, Henkel (Germany) and L'Oréal (France), to assess the intra- and inter-laboratory reproducibility using the model genotoxins, MMC and VB. In Phase 2, three coded compounds, viz. *N*-ethyl-*N*-nitrosourea (ENU), MMC (both genotoxic carcinogens) and cyclohexanone (non-carcinogen and non-genotoxic) were tested at The Procter & Gamble Company (P&G, USA), IIVS (USA) and L'Oréal (France). Good intra- and inter-laboratory reproducibility was obtained for all the chemicals studied, and the genotoxic activity of each chemical was correctly identified in each laboratory.

2. Materials and methods

2.1. Chemicals

All test chemicals (Table 2) were purchased from Sigma–Aldrich. Other reagents such as acetone, ethanol, cytochalasin B (cytoB), trypsin, acridine orange (AO), etc. were obtained as previously reported [7]. Covance UK coded the test articles for the Phase-2 studies and shipped them to the participating laboratories. Once the studies were completed, the results were sent to ECVAM, decoded, and evaluated for validity. Acetone and ethanol were selected as solvents, as was recommended by the Chemical Selection Committee. These solvents are commonly used in dermal carcinogenicity studies in rodents. The use of these solvents allows comparison with previously published results [5–7].

2.2. 3D models and transport

EpiDermTM EPI-200-MNA kits and new maintenance medium (NMM) were obtained from MatTek Corporation (Ashland, MA). The EpiDermTM EPI-200 skin models used in these studies were from human foreskin-derived epidermal keratinocytes from donor 254. The models were shipped overnight by courier and upon receipt were allowed to recover for 24 h (for 48-h experiments) by culturing them in 6-well plates containing 1 ml of NMM at $5 \pm 1\%$ CO₂ and $37 \pm 1^\circ\text{C}$ before use in the RSMN experiments. For 72-h dosing experiments (three doses of test compound), the first dose was applied on the day of arrival approximately 1 h after the medium change.

2.3. RSMN assay

The RSMN assay was conducted according to standard procedures defined by Curren et al. [5], Mun et al. [6] and a consolidated protocol arising during the COLIPA project. The draft OECD guideline for the *in vitro* MN assay was also used as guidance [13]. Training on the RSMN assay and scoring for binucleated and micronucleated cells was conducted during two different workshops at IIVS. EpiDermTM models were re-fed with 1 ml of fresh NMM containing 3 $\mu\text{g}/\text{ml}$ cytoB every 24 h during the chemical exposure period. Three $\mu\text{g}/\text{ml}$ CytoB was selected for these studies based on previous results indicating that this concentration was optimal for the RSMN assay [6]. Test chemicals were dissolved in acetone or ethanol (see Table 2). A dosing volume of 10 μl was applied twice (48-h dosing regimen) or three times (72-h dosing regimen) at 24-h intervals, to the surface center of the EpiDermTM model. At the end of the treatment period, cells were harvested from the EpiDermTM models and mounted onto slides using standard procedures described previously [6]. Briefly, each model was trypsinized by first incubating for 5–15 min in 5 ml Dulbecco's phosphate-buffered saline (DPBS) at room temperature, then for 15 min in 5 ml EDTA (0.1%) at room temperature, then for 15 min in warm (37°C) trypsin-EDTA

solution. After detaching, the tissue and its supporting membrane were transferred to a new well containing 1 ml trypsin-EDTA at 37°C . Cells were released and resuspended into a single cell suspension (1.5 ml) and transferred to a 15-ml conical tube containing 1.0 ml 10% FBS in DMEM (warm) to stop the trypsin activity. Cells were counted for cell density and viability by means of Trypan-blue exclusion.

The cells were centrifuged ($100 \times g$) and the pellet resuspended in 1 ml of warm (37°C) 0.075 M KCl. After approximately 3 min, 3 ml of cold (stored at -15 to -25°C) methanol/acetic acid (3:1) fixative was added to fix the cells, and the cell suspension was centrifuged at $100 \times g$ for 5 min. The supernatant was removed except for the last 100–200 μl , which were used to resuspend the cells. The fixation process was repeated to avoid contamination with salt crystals, which would otherwise interfere with the microscope-slide evaluation. A single drop of the concentrated cell suspension was gently pipetted onto a clean, dry microscope slide (Gold Seal[®], Becton Dickinson & Co.). When the slides (at least one per model) were completely dry, they were immersed in freshly prepared AO solution (final concentration 40 $\mu\text{g}/\text{ml}$ in DPBS) for 2–3 min, immediately rinsed 3 times with DPBS, and then allowed to dry. Stained slides were stored in the dark at 2 – 8°C .

For microscopic analysis, a drop of DPBS and a coverslip were placed over the fixed samples, and the slides were scored with a fluorescence microscope equipped with a blue filter (Opelco, item code CT-11001V2, EX470 BS495 EM515) and a $40\times$ or $60\times$ objective.

2.4. Cytotoxicity and MN assessment

The percentage binucleated (BN) cells, i.e. the frequency of BN cells in 500 cells containing one, two, or more than two nuclei, was evaluated for each model. The relative percentage BN was calculated by comparing the % BN cells for each model with the average % BN of the solvent control. The average relative % BN for a treatment group was the primary endpoint of cytotoxicity measurement for all chemicals, and used to determine the highest concentrations for MN analysis. The maximum cytotoxicity was 60%, based on the draft OECD guideline for the *in vitro* MN assay, which suggests a limit of $55\% \pm 5\%$ cytotoxicity [13]. Cell counts were also obtained for each model.

The number of BN cells with MN (MNBN) was scored with a standard classification method [8,14]. A one-tailed Fisher exact test on the total number of MNBN/total cells analyzed from all models for each treatment was used to determine the statistical significance of the results observed with each test concentration. A result was declared statistically significant if the corresponding *p*-value was <0.05 . Assays were deemed valid if they met the following criteria [7]:

- The yield of viable cells should be higher than $5 \times 10^4/\text{model}$.
- The % BN cells in acetone controls should be greater than 25.
- At least two models per dose level that meet all other criteria.
- At least 500 cells per model should be analyzed for the frequency of mono-nucleated cells, binucleated cells, or cells with more than two nuclei.
- Ideally, 1000 BN cells are analyzed for the presence of MN. Only results from models where at least 500 BN cells are obtained are reported.
- The positive control must cause a statistically significant increase in the % MN compared with the vehicle controls.
- The top dose should be at the limits of solubility, cause a cytotoxicity of at least 60% or be at a maximum concentration of 100 mg/ml.

3. Results and discussion

3.1. Phase 1: transferability of the EpiDermTM RSMN assay methodology to Henkel and L'Oréal – inter-experimental and inter-laboratory comparisons of vehicles, MMC and VB

The purpose of these studies was to confirm that the RSMN assay used by P&G and IIVS could be transferred to other laboratories and more specifically, to those that were not based in the United States. This allowed assessment of possible effects of international transport of the model.

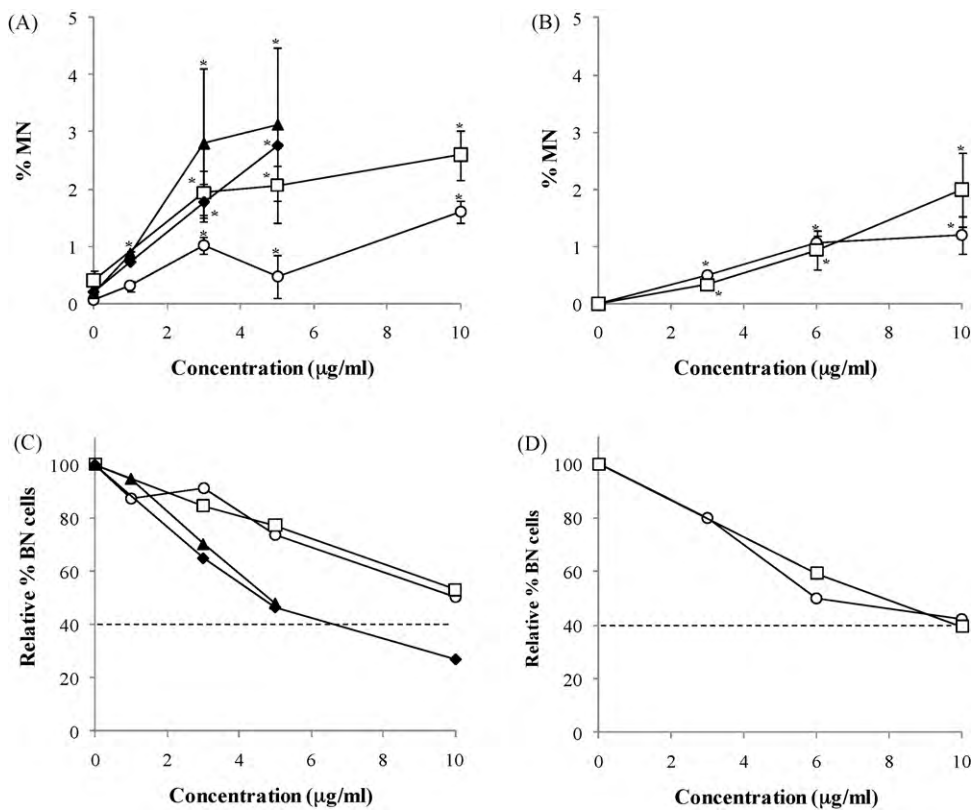


Fig. 1. MMC induced % MN and relative % BN cells in studies conducted at Henkel (A and C) and L'Oréal (B and D). Within each set of results, separate experiments are represented by different symbol shapes. The dotted line shows the 40% cut-off for acceptable % relative BN cell values. Values are mean (\pm SD for % MN only); *significant increase over concurrent vehicle control ($P < 0.05$).

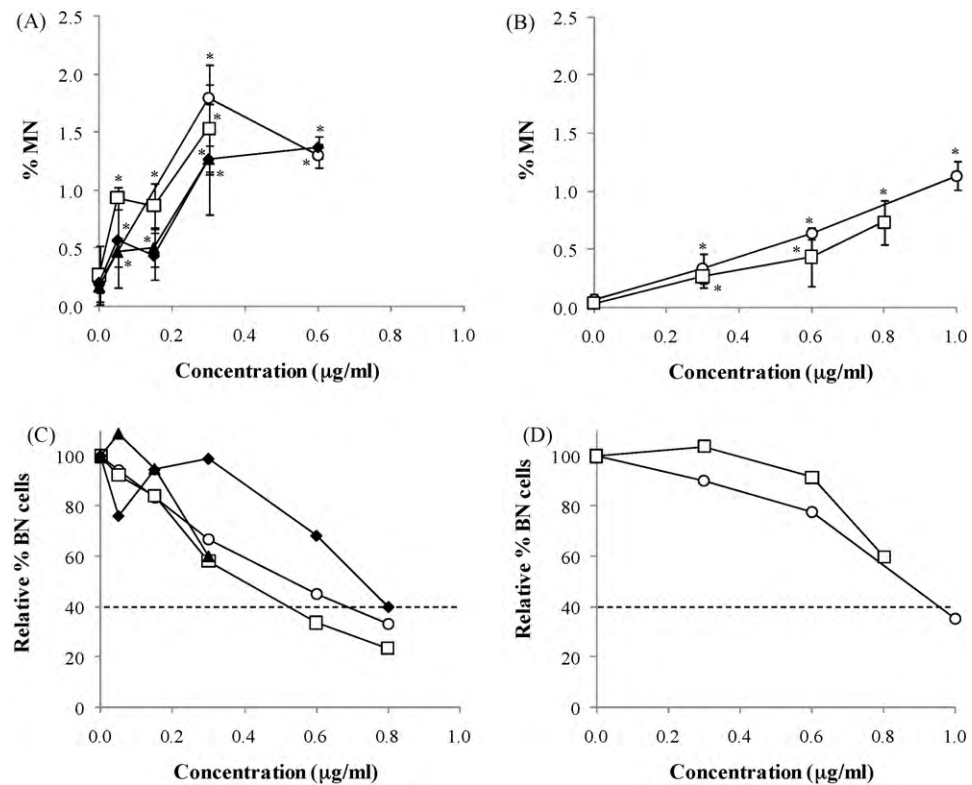


Fig. 2. VB induced % MN and relative % BN cells in studies conducted at Henkel (A and C) and L'Oréal (B and D). Within each set of results, separate experiments are represented by different symbol shapes. The dotted line shows the 40% cut-off for acceptable relative % BN cell values. Values are mean (\pm SD for % MN only); *significant increase over concurrent vehicle control ($P < 0.05$).

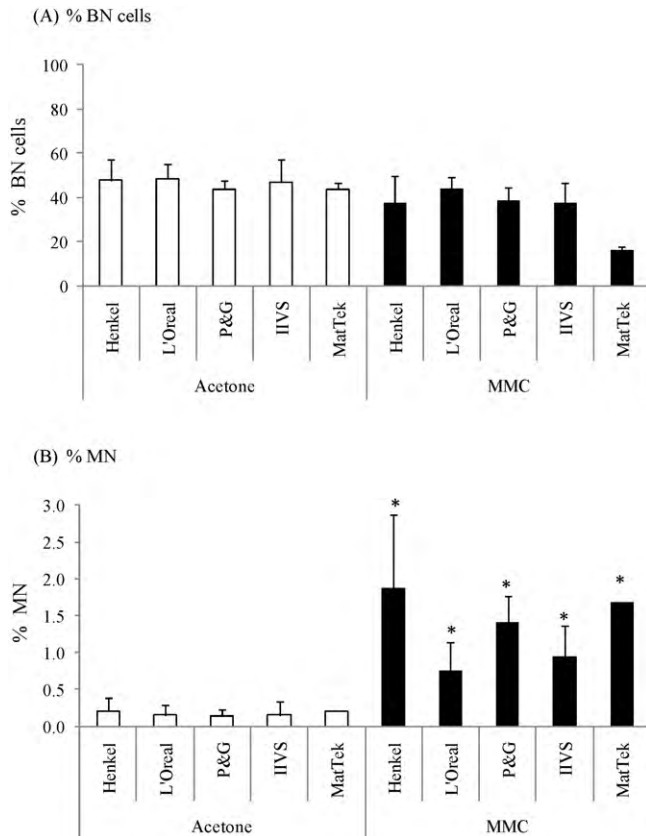


Fig. 3. A comparison of the % BN cells (A) and frequency of MN (B) in models treated with vehicle control (acetone, □) and positive control (3 μ g/ml MMC, ■) for 48 h. Data for MatTek were from Hu et al. [7], error bars for % MN for MatTek are not shown. Values are mean \pm SD; *significant increase relative to laboratory-specific acetone control value ($P < 0.05$).

3.1.1. Comparison of vehicles

The % BN cells and the frequency of MN in acetone- and ethanol-treated models at Henkel and L'Oréal are shown in Table 3. The % BN cells (mean 41–52%) and % MN (mean 0–0.2%) were similar between laboratories for control models treated with either vehicle. A comparison with results from the other laboratories is provided below (Section 3.1.4.1). These results confirm the low MN background previously reported [5–7], which is required for a valid assay. Studies on the suitability of a variety of solvents have been conducted (*manuscript in preparation*).

3.1.2. MMC

There was a dose-dependent increase in the frequency of MN in models treated with MMC at Henkel and L'Oréal (Fig. 1A and B, respectively). Although the Henkel results showed some variability between experiments in the magnitude of the response at 3 and 5 μ g/ml, overall the experiments were reproducibly positive. Results from both laboratories showed a statistically significant increase in the % MN in all experiments at 3 μ g/ml MMC (the standard concentration used as a positive control), although the results for L'Oréal were somewhat lower than previously reported for other laboratories (see Sections 3.1.4.2 and 3.1.4.3). The dose response for toxicity was similar in both laboratories (Fig. 1C and D).

3.1.3. VB

There was a dose-dependent increase in toxicity (decrease in relative % BN cells) and a statistically significant increase in the frequency of MN in models treated with VB at Henkel and L'Oréal (Fig. 2). Intra-laboratory reproducibility was very good as evi-

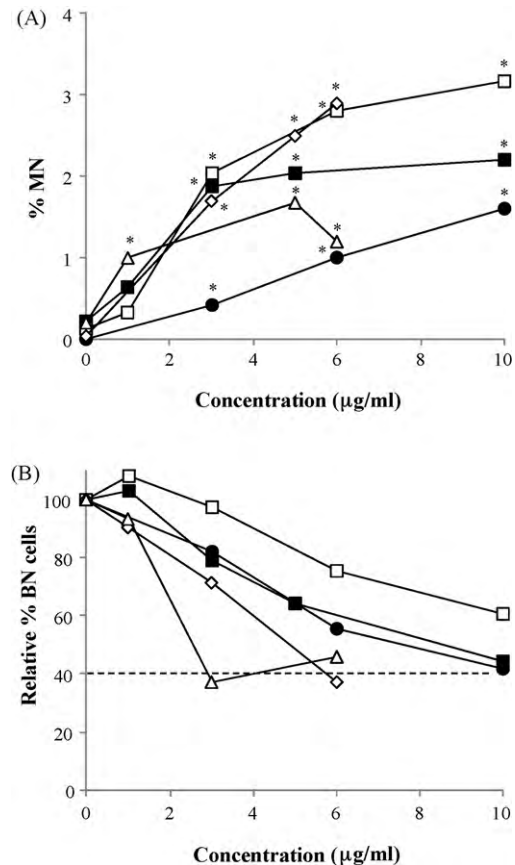


Fig. 4. MMC induced MN (A) and relative % BN cells (B) in studies conducted at 5 different laboratories. Symbols denote different laboratories: Henkel (■), L'Oréal (●), P&G (□), IIVS (◇) and MatTek (△). The dotted line shows the 40% cut-off for acceptable relative % BN cell values. Mean values are shown; *significant increase over concurrent vehicle control ($P < 0.05$).

denced by the close similarity in the dose–response curve between experiments at each laboratory. Both laboratories reproducibly found positive responses for VB, but the magnitude of the response was lower at L'Oréal. The dose–response in toxicity was steep, as has been shown for aneugens in the standard *in vitro* MN assay [15], requiring closely spaced doses (0.2 μ g/ml to 1.0 μ g/ml) in these studies. With steep dose–response curves, there is often variability in the results between laboratories: a higher toxicity and a larger % MN response were observed in studies conducted by Henkel compared with those at L'Oréal, although overall the results were clearly positive in both laboratories.

3.1.4. Comparison with published data

3.1.4.1. Inter-laboratory comparison of vehicle and positive controls. Fig. 3 shows the mean % BN cells and % MN for models treated with vehicle control (acetone) and positive control (3 μ g/ml MMC) in the Henkel and L'Oréal laboratories during the COLIPA transferability studies compared with results reported previously (data from MatTek, IIVS and P&G for models using cells from donor 254 were taken from Hu et al. [7]). There was little variation in the % BN cells counted for acetone- and MMC-treated models in each laboratory (Fig. 3A), with the exception of MatTek, in which 3 μ g/ml MMC had greater cytotoxicity than observed in other laboratories. The average % MN measured in acetone-treated models was also comparable between laboratories (Fig. 3B). There was some variation in the % MN in models treated with 3 μ g/ml MMC, but all values were statistically significantly higher than that of the corresponding control-treated models (laboratory by laboratory comparison).

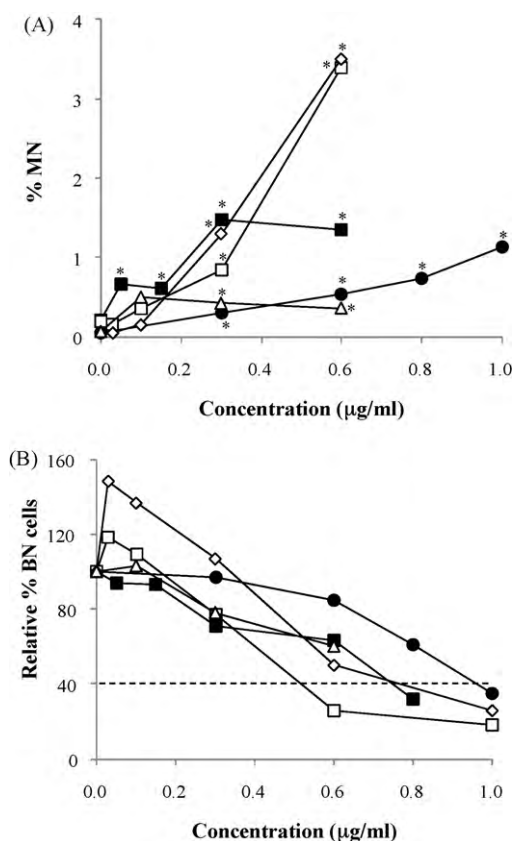


Fig. 5. VB induced MN (A) and relative % BN cells (B) in studies conducted at 5 different laboratories. Symbols denote different laboratories: Henkel (■), L'Oréal (●), P&G (□), IIVS (◇) and MatTek (△). The dotted line shows the 40% cut-off for acceptable relative % BN cell values. Mean values are shown; *significant increase over concurrent vehicle control ($P < 0.05$).

Differences in solubilization of MMC can result in variation in the MMC response [7].

3.1.4.2. Inter-laboratory comparison of dose responses to MMC. The mean % MN and relative % BN cells for MMC from the studies conducted at Henkel and L'Oréal described above are shown with results from P&G, IIVS and MatTek taken from Hu et al. [7], to allow comparison among five laboratories conducting the assay over time (Fig. 4). There was a fair agreement between the five laboratories, with toxicity observed at 3 µg/ml MMC and above, and statistically significant increases in % MN at 3 µg/ml MMC and higher. The % MN counted at L'Oréal was lower than that reported by the four other laboratories, but this was later found to largely be due to differences in scoring (e.g., MN in RSMN in EpiDerm™ can be small and closely associated with the main nuclei) and the solubilization procedure for MMC. After additional harmonization and training, including analysis of a standard set of slides, L'Oréal re-analyzed their slides with harmonized criteria, which resulted in a higher % MN, comparable with that found in the other laboratories.

3.1.4.3. Inter-laboratory comparison of dose responses to VB. The mean % MN and relative % BN after treatment with VB from the studies conducted at Henkel and L'Oréal described above are shown with results from P&G, IIVS and MatTek taken from Hu et al. [7] (Fig. 5; NB: the result for VB 0.1 µg/ml in the IIVS data in Hu et al. [7] should be 0.15% MN, not 1.5% as shown in the paper). Again there was good agreement between the laboratories, with each laboratory obtaining statistically significant increases in the % MN at 0.3 µg/ml VB and above. As described above for MMC, the outcome of the MN scoring at L'Oréal and MatTek was lower than that in the three other laboratories.

3.2. Phase 2: reproducibility of the RSMN assay – testing of three coded chemicals

P&G, IIVS and L'Oréal each tested three chemicals that were coded (different codes for each laboratory), i.e. a blind trial. These

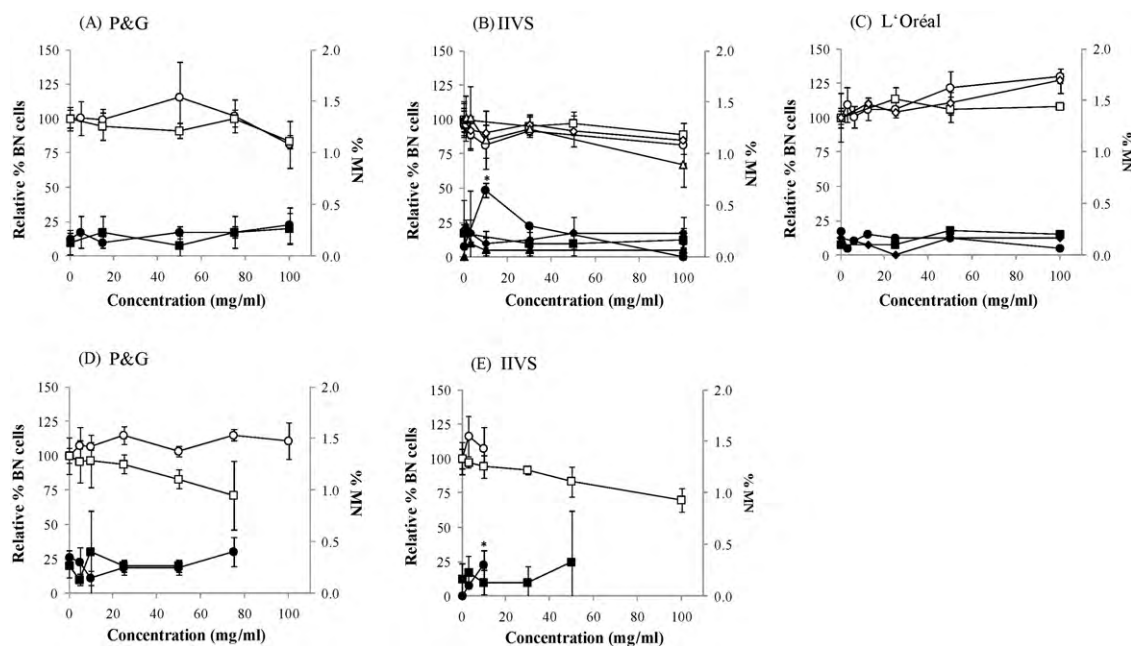


Fig. 6. A comparison of the genotoxic response of models incubated at P&G (A and D), IIVS (B and E) and L'Oréal (C) for 48 h (A–C) and 72 h (D and E) with cyclohexanone as the coded chemical. Open symbols denote the relative % BN cells (left axis) and closed symbols denote % MN (right axis). Within each set of results, separate experiments are represented by different symbol shapes. Values are mean \pm SD; *significant increase over concurrent vehicle control ($P < 0.05$).

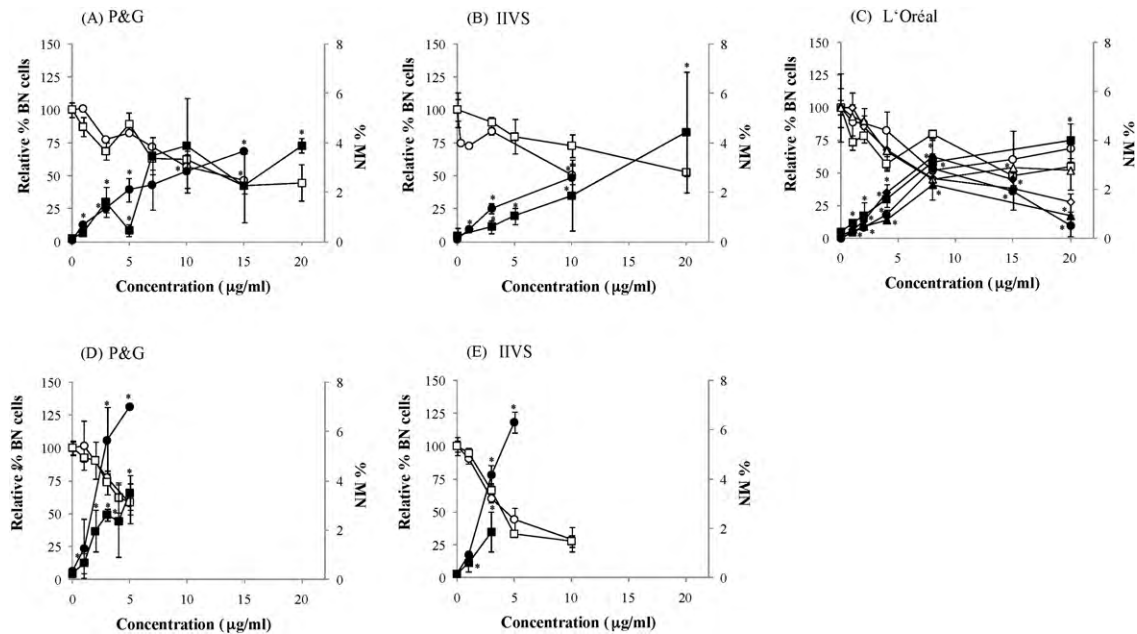


Fig. 7. A comparison of the genotoxic response of models incubated at P&G (A and D), IIVS (B and E) and L'Oréal (C) for 48 h (A–C) and 72 h (D and E) with MMC as a coded chemical. Open symbols denote the relative % BN cells (left axis) and closed symbols denote % MN (right axis). Within each set of results, separate experiments are represented by different symbol shapes. Values are mean \pm SD; *significant increase over concurrent vehicle control ($P < 0.05$).

compounds were cyclohexanone (non-carcinogenic and non-genotoxic), MMC and ENU (both genotoxic carcinogens) (Table 2). Each laboratory was instructed to use a maximum concentration of 10% (100 mg/ml) for these studies (decision of the Steering Committee). Based on initial results indicating that a longer exposure time may be needed in the RSMN assay for chemicals requiring metabolism [9], P&G and IIVS conducted studies with a 72-h exposure regimen in addition to the standard 48-h exposure regimen to determine if there was any impact of the extended treatment of

chemicals that do not require metabolism on either the cytotoxic or the genotoxic response.

3.2.1. Cyclohexanone

As shown in Fig. 6, cyclohexanone generally induced little or no toxicity in these studies up to the maximum concentration of 100 mg/ml. The overall results in each laboratory were concluded to be negative at both 48 h (three laboratories) and 72 h (two laboratories). In one IIVS experiment at 48 h, a single dose of 10 mg/ml

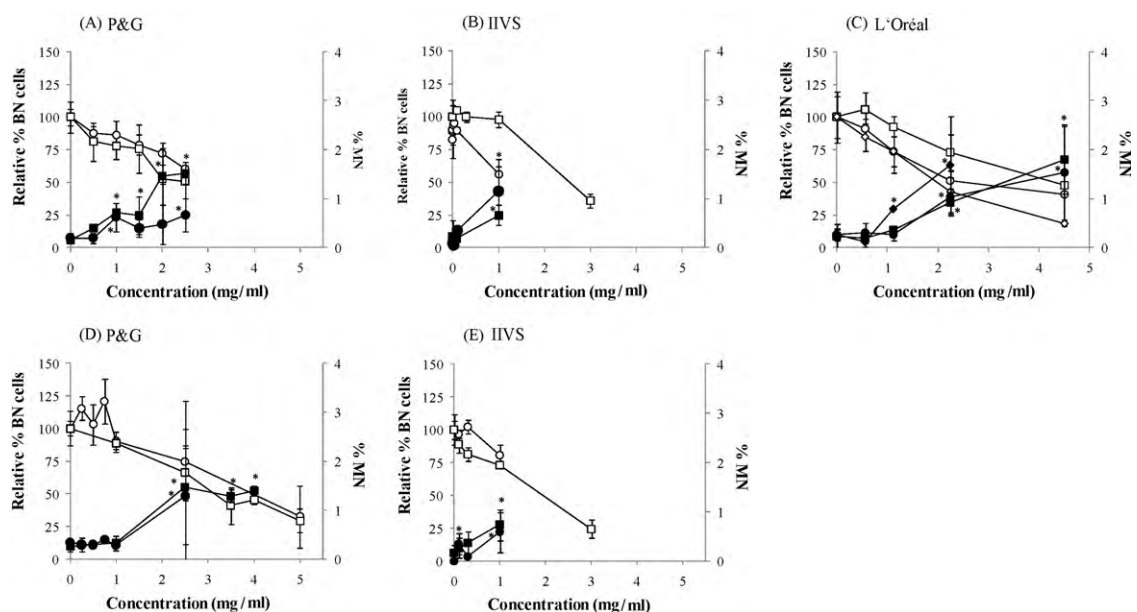


Fig. 8. A comparison of the genotoxic response of tissues incubated at P&G (A and D), IIVS (B and E) and L'Oréal (C) for 48 h (A–C) and 72 h (D and E) with ENU as the coded chemical. Open symbols denote the relative % BN cells (left axis) and closed symbols denote % MN (right axis). Within each set of results, separate experiments are represented by a different symbol shapes. Values are mean \pm SD; *, significant changes ($P < 0.05$) (E).

resulted in a statistically significantly increased % MN ($0.65 \pm 0.07\%$) compared with $0.10 \pm 0.14\%$ in the control (Fig. 6B) whereas no increase at any dose level was seen in the other three experiments up to 100 mg/ml. Likewise, a small, but significant increase in % MN was observed at IIVS in skin models treated for 72 h at IIVS at 10 mg/ml was observed in experiment 1 ($0.30 \pm 0.14\%$ compared to $0.00 \pm 0.00\%$ in control models (Fig. 6E)). However a repeat study extending to higher concentrations was negative. Therefore, cyclohexanone was considered negative in the IIVS studies due to a lack of a dose-dependent, reproducible increase in % MN. This conclusion is supported by the results in all the studies in the other laboratories.

3.2.2. MMC

Results for MMC tested as a coded chemical are shown in Fig. 7. A similar dose-dependent increase in toxicity was observed in all three laboratories for the 48-h experiments and in two laboratories for the 72 h experiments. MMC induced a statistically significant increase in MN in all the studies at 48 h and 72 h. Overall results for MMC – when tested blind – were similar to the results from Phase 1 where MMC was tested uncoded, and to previous reports [7]. In the Phase-2 studies at L'Oréal, a higher response in % MN to MMC was observed compared with the Phase-1 studies in the same laboratory and a dose response similar to that found in the other two laboratories was seen. This is likely due to harmonization of scoring and MMC solubilization.

3.2.3. N-Ethyl-N-nitrosourea

ENU induced a statistically significant increase in % MN in all studies at both 48 and 72 h (Fig. 8). A statistically significant increase in % MN for ENU in the same dose range of 1–3 mg/ml was reported in Mun et al. [6]. Concentrations of ENU above 1 mg/ml tested at IIVS were very toxic (<40% relative % BN cells) and, therefore, scoring was not carried out in models treated with 3 mg/ml or 9 mg/ml.

3.2.4. Effect of treatment regimen: 48 h versus 72 h

Fig. 9 shows the effect of prolonged treatment duration, from 48 h to 72 h, on the % BN cells and % MN in vehicle-control (acetone) models and in 3 µg/ml MMC-treated models. The longer treatment duration did not affect the % BN cells in acetone- and MMC-treated models or the % MN in acetone-treated models. However, there appeared to be an increase in the % MN in models treated with 3 µg/ml MMC when the duration of treatment was increased from 48 h to 72 h (Fig. 9B). This was also seen in the responses across a wider dose range of MMC, as shown in Fig. 7. MMC was more toxic to models treated for 72 h compared with 48 h, as evidenced by the fact that at 72 h the concentrations higher than 5 µg/ml exceeded the toxicity limit of 60% in P&G and IIVS studies, whereas concentrations up to 20 µg/ml could be tested in a 48 h treatment. The magnitude of the MN response towards MMC was higher for the 72 h treatment time compared with 48 h in two studies, one at P&G and one at IIVS.

The toxicity and % MN in models treated with cyclohexanone (Fig. 6) and ENU (Fig. 8), which were found to be negative and positive for genotoxicity, respectively, were unaffected by the treatment duration.

3.2.5. Effect of transport

The results described above indicate that the RSMN assay in EpiDerm™ was successfully transferred to the European-based laboratories, Henkel and L'Oréal. The success of the assay was partly dependent on establishing a process for the laboratories to receive the models by overnight shipment. Models were shipped on a Monday and arrived at the European laboratories late Tuesday

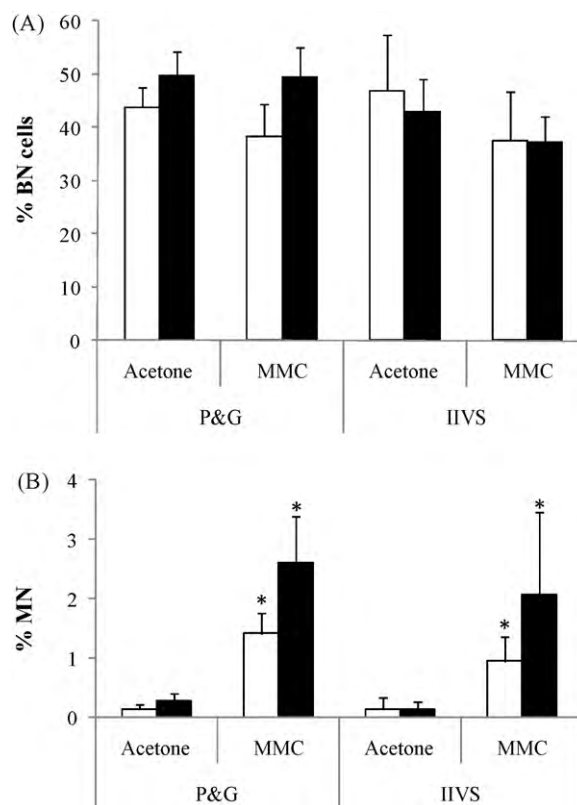


Fig. 9. Effect of treatment time on the % BN cells (A) and % MN (B) in models treated with vehicle (acetone) and a positive control (3 µg/ml MMC). Tissues were treated for 48 h (□) or 72 h (■) in two laboratories. Values are mean \pm SD; *significant increase over concurrent vehicle control ($P < 0.05$); $n = 10$ –18 for the 48 h time point and $n = 8$ for the 72 h time point.

afternoon. Shipments in the United States are also made on Monday and arrive in the morning on Tuesdays. On occasion when the transport time was delayed by more than one day, the quality of the models and number of cells recovered was significantly lower.

4. Conclusions

The results from this COLIPA/ECVAM pre-validation project extend previous reports on this novel *in vitro* RSMN assay in the EpiDerm™ model. Phase-1 transferability and optimization studies with two model genotoxins, MMC and VB, were reproducible within and between laboratories in Europe. As with previous findings, MMC and VB induced a clear and dose-dependent increase in the frequency of MN. In Phase-2 studies, three coded chemicals were correctly and reproducibly identified as either positive or negative in all three laboratories. These data support the conclusion that the RSMN assay in EpiDerm™ is a valuable *in vitro* method for genotoxicity assessment of dermally applied chemicals. Phase-3 studies to increase the domain of chemicals tested and further evaluate reproducibility are underway. It is hoped that other laboratories investigate the RSMN assay in EpiDerm™ and other RS models in order to add to our understanding of differences in performance of the assay across a wide variety of chemicals with different mechanisms of action and using RS models with differing DNA-repair mechanisms. Investigation of the potential utility of more complex models, such as full-thickness skin models, will be included among other important studies.

Conflict of interest statement

No conflict of interest.

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