



Intralaboratory and interlaboratory evaluation of the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay

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ABSTRACT

A novel *in vitro* human reconstructed skin micronucleus (RSMN) assay has been developed using the EpiDerm™ 3D human skin model [R. D. Curren, G. C. Mun, D. P. Gibson, and M. J. Aardema, Development of a method for assessing micronucleus induction in a 3D human skin model EpiDerm™, *Mutat. Res.* 607 (2006) 192–204]. The RSMN assay has potential use in genotoxicity assessments as a replacement for *in vivo* genotoxicity assays that will be banned starting in 2009 according to the EU 7th Amendment to the Cosmetics Directive. Utilizing EpiDerm™ tissues reconstructed with cells from four different donors, intralaboratory and interlaboratory reproducibility of the RSMN assay were examined. Seven chemicals were evaluated in three laboratories using a standard protocol. Each chemical was evaluated in at least two laboratories and in EpiDerm™ tissues from at least two different donors. Three model genotoxins, mitomycin C (MMC), vinblastine sulfate (VB) and methyl methanesulfonate (MMS) induced significant, dose-related increases in cytotoxicity and MN induction in EpiDerm™ tissues. Conversely, four dermal non-carcinogens, 4-nitrophenol (4-NP), trichloroethylene (TCE), 2-ethyl-1,3-hexanediol (EHD), and 1,2-epoxydodecane (EDD) were negative in the RSMN assay. Results between tissues reconstructed from different donors were comparable. These results indicate the RSMN assay using the EpiDerm™ 3D human skin model is a promising new *in vitro* genotoxicity assay that allows evaluation of chromosome damage following “*in vivo*-like” dermal exposures.

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1. Introduction

Currently, studies using laboratory animals such as the *in vivo* bone marrow MN assay and the *in vivo* unscheduled DNA synthesis (UDS) assay are used to further evaluate the genotoxic potential of chemicals that are positive in *in vitro* genotoxicity assays. According to the EU 7th Amendment to the Cosmetics Directive [2], starting in 2009, *in vivo* assays for genotoxicity will be banned for cosmetics ingredients. Without *in vivo* genotoxicity assays, industry and regulators will have to rely solely on the results of *in vitro* genotoxicity tests. However these tests have been shown to produce unacceptably high rate of positive results that are not confirmed in *in vivo* genotoxicity and/or rodent carcinogenicity tests, i.e. “false” or “irrelevant” positive results [3]. In fact 75–90% of rodent non-carcinogens were found to be positive in one or more of the standard *in vitro* genotoxicity assays [3]. Thus, relying only on results from *in vitro*

genotoxicity assays would severely impact the ability to market potentially safe and beneficial new cosmetic products. To address this issue, we developed a micronucleus assay using the EpiDerm™ 3D reconstructed human skin model as a potential replacement for an *in vivo* genotoxicity assay to support the regulatory safety evaluation for cosmetic products and other dermally exposed chemicals [1].

Based on the success of our initial studies, we expanded our work to refine and improve the methods, and increase the size of the 3D EpiDerm™ RSMN assay database to begin to address predictive capability [4]. In this paper we have address several key elements of assay validation, namely intralaboratory and interlaboratory reproducibility. Because primary foreskin derived human epidermal keratinocytes are used in the construction of the EpiDerm™ cultures (<http://www.mattek.com>), it is important to understand the effect of tissue constructs generated from different donors on the overall performance of this assay. In this study, 3D EpiDerm™ tissues generated using cells from four different donors were used to assess variability of the model prepared from different donors. The standard testing protocol defined by Curren et al. [1] and

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Table 1

List of compounds tested.

Compound	Abbreviation	CAS no.	Vehicle	Supplier	3D RSMN assay result ^a
Genotoxins					
Mitomycin C	MMC	50-07-7	acetone ethanol or	Sigma–Aldrich	+
Vinblastine sulfate	VB	143-67-9	acetone	Sigma–Aldrich	+
Methyl methanesulfonate	MMS	66-27-3	acetone	Sigma–Aldrich	+
Dermal non-carcinogens					
4-Nitrophenol	4-NP	100-02-7	acetone	Sigma–Aldrich	—
Trichloroethylene	TCE	79-01-6	acetone	Sigma–Aldrich	—
2-Ethyl-1,3-hexanediol	EHD	94-96-2	acetone	Acros Organics	—
1,2-Epoxydodecane	EDD	2855-19-8	acetone	Sigma–Aldrich	—

^a + = positive, $p < 0.05$; – = negative.

further refined by Mun et al. [4] was utilized in three laboratories: P&G (Procter & Gamble Company), IIVS (Institute for In Vitro Sciences, Inc.) and MatTek (MatTek Corporation) to evaluate seven test chemicals: three model genotoxins (MMC, VB and MMS) and four dermal non-carcinogens (4-NP, EDD, TCE and EHD) (chemical selection reviewed in accompanying paper, Mun et al. [4]).

Overall, reproducible isolation of cells and cell yield along with consistently low background MN frequencies (an average of 0.08%) have been obtained across the tissues constructed from different cell donors in the three laboratories. Comparable MN levels were observed in tissues constructed from the different donor tissues with positive results for the three genotoxins and negative results for all four dermal non-carcinogens in three laboratories. These results demonstrate that the current protocol for the EpiDerm™ 3D human RSMN assay is robust and gives reproducible results within and between different laboratories.

2. Materials and methods

2.1. Chemicals

All the chemicals tested (Table 1) were purchased from Sigma–Aldrich or Acros Organics. Other reagents such as acetone, ethanol, cytochalasin B (cytoB), trypsin, acridine orange (AO) etc. were obtained as previously reported [1].

2.2. Tissue constructs

EpiDerm™ EPI-200-MNA kits, supplied with new maintenance medium (NMM), were obtained from MatTek Corporation (Ashland, MA). The NMM contains keratinocyte growth factor and allows acceptable differentiated morphology of the EpiDerm™ EPI-200 tissue for at least five days upon receipt by end users. The EpiDerm™ EPI-200 tissue constructs are 0.64 cm² human skin equivalents resembling the normal human epidermis (<http://www.mattek.com>). Experiments described here were conducted with EpiDerm™ cultures constructed using foreskin derived human epidermal keratinocytes from four different donors. All tissues used in studies at MatTek Corporation went through mock shipment conditions to insure the comparability of the tissues to those shipped to IIVS and P&G. Upon receipt (at IIVS or P&G) or overnight storage (at MatTek), tissues were cultured overnight 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.

2.3. RSMN assay

According to the standard procedures defined by Curren et al. [1] and Mun et al. [4], EpiDerm™ tissue constructs were refed with 1 ml of fresh NMM containing 3 $\mu\text{g}/\text{ml}$ cytoB approximately every 24 h during the 48 h chemical exposure period. Test chemicals were dissolved in acetone or ethanol, and a dosing volume of 10 μl was applied twice, 24 h apart, carefully with a micropipette to the surface center of the EpiDerm™ tissue. The standard procedures were used for harvesting cells from the EpiDerm™ tissues, for measuring cell yield and for processing cells to prepare slides for the cell proliferation and MN analyses. Intralaboratory reproducibility of the RSMN at IIVS is addressed in Mun et al. [4]. Therefore only one representative study that meets all the appropriate criteria for a valid study including appropriate levels of toxicity from IIVS is shown in this paper, with the exception of MMC where studies from two different donors are provided.

2.4. Cytotoxicity and MN assessment

The % binucleate (BN) cells, as calculated by the frequency of BN cells in 500 cells containing one, two, or more than two nuclei, was evaluated for each tissue. The % relative BN was calculated by comparing the %BN level for each tissue to the average % BN of the solvent control. The average of the % relative BN for a treatment group was the primary endpoint of cytotoxicity measurement for all seven chemicals, and used to determine the highest concentrations for MN analysis of four chemicals (MMC, VB, MMS and 4-NP). The percentage of relative cell yield was calculated by comparing the cell yield of chemical treated tissues to its corresponding control tissues, and for some chemicals (EDD, TCE and EHD) this was a more sensitive indicator of cytotoxicity than reduction in the % relative BN. The highest concentrations of a test chemical reported here for analysis of MN produced 50–70% cytotoxicity (by either decreased %BN or decreased % cell yield) compared to controls. This toxicity range was consistent with the draft OECD guideline 487 [5] for the *in vitro* micronucleus assay in place at the time these studies were conducted (<http://www.oecd.org>) though a lower level of toxicity (around 50%) now appears to be an acceptable limit. All genotoxic chemicals evaluated in the studies below would also have been positive if a top concentration was selected at around 50% toxicity.

The number of BN cells with micronuclei (MNBN) was scored using a standard classification method [6]. The frequency of MN per 1000 BN cells (when possible) was generated for each scorable slide/tissue. The program StatXact-Turbo (Cytel Software Corporation, Cambridge, MA) was used for statistical analysis. A one-tailed Fisher exact test was used to determine the statistical significance of each test concentration. A result was declared statistically significant if the corresponding p -value was < 0.05 .

3. Results and discussion

3.1. Reproducibility of the EpiDerm™ RSMN methodology

As shown in Fig. 1A, solvent control tissues in all three labs typically yielded $1\text{--}5 \times 10^5$ cells, with an average cell yield of around 2.5×10^5 cells/tissue. The variation in the range of cells from tissues is to be expected in this type of primary cell tissue model, especially as labs start using the methods. Previous studies conducted at IIVS [1] yielded an average of 2.3×10^5 cells/tissue which is comparable to the average reported in studies conducted at P&G (3.7×10^5 cells/tissue) and MatTek (2.8×10^5 cells/tissue) as shown in Table 2. Importantly, this cell yield provides enough cells to prepare at least two slides and sometimes up to 4 slides with a single drop of cell suspension per slide from each tissue. This procedure yields sufficient cells for the analysis of MN.

After 48 h incubation with 3 $\mu\text{g}/\text{ml}$ cytoB, 22–66% BN cells were obtained in acetone solvent control tissues across the laboratories with a mean of 44% across all three laboratories (Fig. 1B, Table 2). This value is in line with the levels reported by Curren et al. [1] of 40–55% BN cells. Most studies were conducted with tissue donor 254 and the range of BN cells was 25–56%. The range for donor 4F1188 was 48–66%, and too few studies were conducted with donors 219 and 926 to evaluate a range of responses. Statistical analysis of the %BN cell level in RSMN experiments from all three laboratories indicated that 25% BN level in the solvent control tissues represents the lower end of the 95% confidence interval.

The %MN in acetone solvent control treated tissues ranged from 0 to 0.5% with an average around 0.08% among the three labora-

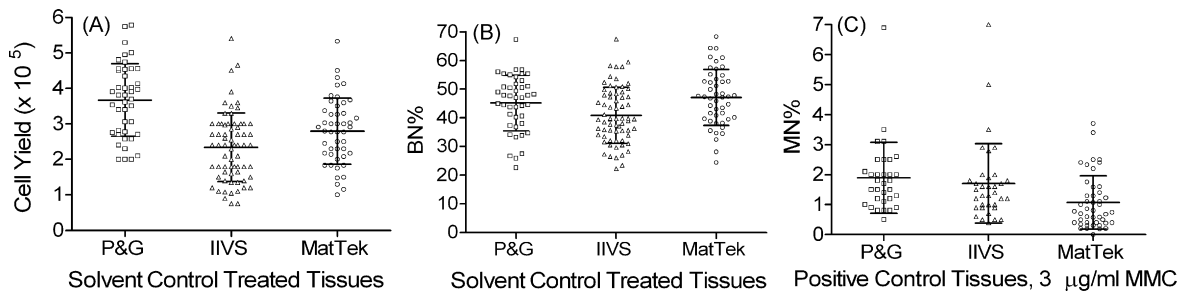


Fig. 1. Comparison of solvent and positive control treated tissues in EpiDerm™ 3D RSMN assays from P&G, IIVS and MatTek studies. (A) Cell recovery of solvent control tissues, (B) percentage of BN in solvent control treated tissues and (C) percentage of MNBN cells in 3 µg/ml MMC treated tissues.

Table 2
Summary of cell yield from solvent control tissues and percentage of binucleated cells in 3D EpiDerm™ RSMN assay.

	Acetone control cell count (×10 ⁵ cells)			Acetone control binucleation level (%)		
	P&G	IIVS	MatTek	P&G	IIVS	MatTek
No. of tissues ^a	43	67	47	43	67	47
Mean ± SD	3.7 ± 1	2.3 ± 1.0	2.8 ± 0.9	45.2 ± 9.7	40.9 ± 9.7	47.1 ± 9.8
Maximum	5.8	5.4	5.3	67.3	67.4	68.28
Minimum	2	0.8	1	22.6	22.3	24.4

^a Total number of EpiDerm™ tissues from a combination of all donors tested in each laboratory.

tories (Table 3). These results confirm the low background %MN (average 0.05%) reported previously [1] and further establish the reproducibility of the methods. We routinely use 3 µg/ml MMC as our positive control in the assay and average %MN results were 1.89% at P&G, 1.07% at MatTek and 1.7% at IIVS with an overall average among the 3 laboratories of 1.58% (Table 3 and Fig. 1C). These results are similar to those reported by Curren et al. [1] of 1.8% following topical exposure of MMC in acetone.

3.2. Parameters for a valid assay

Based on the results for positive and solvent control treated cultures, several parameters which are important to the successful conduct of the assay are defined. It is especially important to define these parameters for this type of model since primary cell-based tissue model inherently have more sources of variation than clonal cell lines. In addition, suggested criteria for a valid assay, taking into account the draft OECD guidelines for the *in vitro* micronucleus assay, are provided. These parameters will need to be reviewed as data from more laboratories are generated following the procedures for the RSMN in EpiDerm™ tissues. The studies reported below meet these characteristics.

1. Cell yield: Based on the range and average yield of cells/tissue described above, a minimum of 5 × 10⁴ cells/tissue in the solvent control is needed for a tissue to be considered valid in the RSMN assay. If fewer cells are obtained in the solvent control tissues, the lab's procedures should be reviewed. Importantly, lower cell yields would impact the ability to obtain the recommended number of cells for analysis.

- 2. Percentage of BN cells: 25% BN cells (lower end of the 95% confidence interval) in acetone treated control tissues is recommended as the lowest limit for a tissue to be considered acceptable for analysis. If the %BN in any solvent control tissues in a particular study is less than 25%, it likely indicates a technical issue or problem with the EpiDerm™ tissue. This is also a practical limitation since any test chemical-induced toxicity will result in too few BN cells for analysis when the control values are too low. To insure that a study is valid, a preliminary assessment of the %BN in solvent controls can be performed, and if the results are valid, the slides are coded and included in the blinded analysis of the rest of the slides.
- 3. Number of tissues: Because of variability in these tissues especially after treatment with a toxic chemical, and to insure obtaining sufficient numbers of cells for analysis (see below), we recommend at least 3 tissues be used per treatment group. Results from at least 2 valid tissues must be available for a treatment group to be considered a valid dose.
- 4. Percentage of MNBN in solvent controls: At this point in time, there are no criteria for a valid %MNBN in solvent controls. Each lab needs to establish its own historical control. The range in our laboratories is from 0 to 0.5% with an average around 0.08%. If a higher %MN frequency is obtained, the slide should be rescored to check scoring accuracy.
- 5. Analysis of cell proliferation: At least 500 cells must be scored for the percentage of cells containing one, two, or more than two nuclei per tissue. As described in Mun et al. [4], only cells with red cytoplasmic acridine orange (AO) staining are analyzed. Differentiated cells with green cytoplasmic AO staining are not analyzed. If a culture has less than 500 analyzable cells and cells are of poor quality due to test article toxicity, it is listed as "T" for

Table 3
Summary of MN frequency in solvent control tissues and positive control tissues in 3D EpiDerm™ RSMN assay.

	Acetone solvent control MN frequency (%)			3 µg/ml MMC positive control % micronucleated binucleated cells		
	P&G	IIVS	MatTek	P&G	IIVS	MatTek
No. of tissues ^a	43	67	47	32	35	45
Mean ± SD	0.07 ± 0.11	0.1 ± 0.12	0.07 ± 0.09	1.89 ± 1.18	1.71 ± 1.32	1.07 ± 0.9
Maximum	0.4	0.5	0.4	6.9	7	3.7
Minimum	0	0	0	0.5	0.4	0

^a Total number of EpiDerm™ tissues from a combination of all donors tested in each laboratory.

Table 4

Summary of mitomycin C results using tissue constructed from three different donors tested in three different laboratories.

Compound	Donor	Concentration ($\mu\text{g/ml}$)	Applied on EpiDerm™ ($\mu\text{g/cm}^2$)	Average binucleation% \pm SD (% decrease solvent control)			% Micronucleated bicleated cells		
				P&G	IIVS	MatTek	P&G	IIVS	MatTek
Mitomycin C	254	0	0	25.1 \pm 2.2 (0)	37.6 \pm 6 (0)	43.7 \pm 3 (0)	0.12	0.04	0.2
		1	0.03	27.1 \pm 3.0 (–8)		40.8 \pm 13 (7.5)	0.33		1.0**
		3	0.09	24.4 \pm 1.6 (2.7)	26.8 \pm 4 (28.9)	16.3 \pm 1.3 (62.9)	2.03**	1.7**	1.68**
		6	0.19	18.9 \pm 2.5 (24.8)	13.9 \pm 1.3 (62.9)	20.1 \pm 0.7 (54.4)	2.8**	2.9**	1.2**
		10	0.31	15.2 \pm 1.9 (39.4)			3.16**		
		15	0.47	9.6 \pm 0.6 (61.8)			3.11**		
	4F1188	0	0	47.7 \pm 2.7 (0)	61.2 \pm 5.4 (0)	55.9 \pm 8.0 (0)	0.13	0	0.16
		1	0.03	44.7 \pm 4.2 (6.4)	59.7 \pm 4.4 (2.5)	52.6 \pm 0.9 (5.8)	1.15**	0.1	0.95**
		3	0.09	41.0 \pm 7.6 (14.3)	64.3 \pm 4.1 (–5.1)	47.2 \pm 5.8 (15.6)	3.48**	0.2*	0.93**
		6	0.19	32.1 \pm 2.8 (32.3)	50.6 \pm 0.6 (17.4)	38.7 \pm 1.4 (30.1)	4.25**	0.57**	0.87**
		10	0.31	16.7 \pm 4.1 (65)	40.7 \pm 6.9 (33.6)		5.03**	1.13**	
		15	0.47	11.9 \pm 4.2 (75)			3.75**		
	219	0	0			58 \pm 8.4 (0)			0.1
		1	0.03			53.9 \pm 6.9 (7.1)			1.3**
		3	0.09			60.7 \pm 5.8 (–4.7)			0.3
		6	0.19			43.7 \pm 4.1 (24.6)			3.2**

* $p < 0.05$.** $p < 0.01$.

toxic and this culture is averaged into the calculation of toxicity as 100% toxicity. The analysis for toxicity is performed in a separate analysis from the analysis for MNBN cells to avoid bias in cell selection for quantitating MN. For toxicity, cells with reasonably intact cytoplasmic membranes are analyzed. In contrast, for the analysis of MN cells, the cytoplasm should be retained and well spread to allow detection of MN. The relative %BN for each treatment condition is calculated and only tissues showing less than 70% cytotoxicity compared to the average %BN of the solvent controls are counted for MN frequency. We are now using a 55% cytotoxicity limit (% relative BN or cell counts) in this assay based on the latest draft OECD guideline for the invitro micronucleus assay (5).

- Numbers of cells analyzed for MN: 1000 BN cells (with red cytoplasmic AO staining) are analyzed per tissue to determine the frequency of MNBN. Only results from tissues that had at least 500 analyzable BN cells are reported. If less than 500 analyzable BN cells are present, the tissue is considered “not scorable” and is not included in the mean calculation of %MNBN for that dose.
- Positive control: The positive control must result in a statistically significant increase in MN% level. MMC (Sigma M4287 with NaCl, cell culture tested) at 3 $\mu\text{g/ml}$ in acetone has been found to give the most reproducible results and is now typically used in our labs.

3.3. Evaluation of model chemicals

3.3.1. Mitomycin C

As shown in Table 4 and Fig. 2, dose dependent decreases in the %BN cells and dose dependent statistically significant increases

in MN induction at multiple concentrations were observed in all donor tissues from the studies of all three laboratories after topical application of MMC, a known direct acting crosslinking genotoxin. In the IIVS experiment conducted with tissues derived from donor 254 cells, 60% toxicity was observed at 6 $\mu\text{g/ml}$ MMC as previously reported by Curren et al. [1]. At 6 $\mu\text{g/ml}$ MMC, a range of toxicity from 17 to 62% was obtained in the three laboratories using tissues comprised of cells derived from donors 254, 4F1188 and 219. Though significant increases in MMC induced MN were observed in all three laboratories, there was some variability in effects observed within and between laboratories that may be due to differences in completeness of solubilizing the MMC. Revised procedures for preparing homogenous MMC dosing solutions are described in Mun et al. [4]. 3 $\mu\text{g/ml}$ MMC is typically used as the positive control concentration for our assays since it induces a statistically significant increase in MN, but with low toxicity.

3.3.2. Vinblastine sulfate

Table 5 and Fig. 3 summarize the results for VB tested in all three laboratories. Results shown for IIVS were previously reported [1]. Dose dependent decreases in the %BN cells and dose dependent and statistically significant increases in MN induction were observed in all donor tissues from the studies of all three laboratories after topical application of VB. In the studies conducted at P&G, ethanol was used as the solvent for VB to directly compare to the original IIVS VB study [1]. The level of toxicity and MN induction were nearly identical from studies at P&G and IIVS. Since ethanol was previously found to sometimes induce separation of the tissue from the support membrane, i.e. “blistering” [1] (not seen in subsequent studies), MatTek performed studies with VB using acetone as the

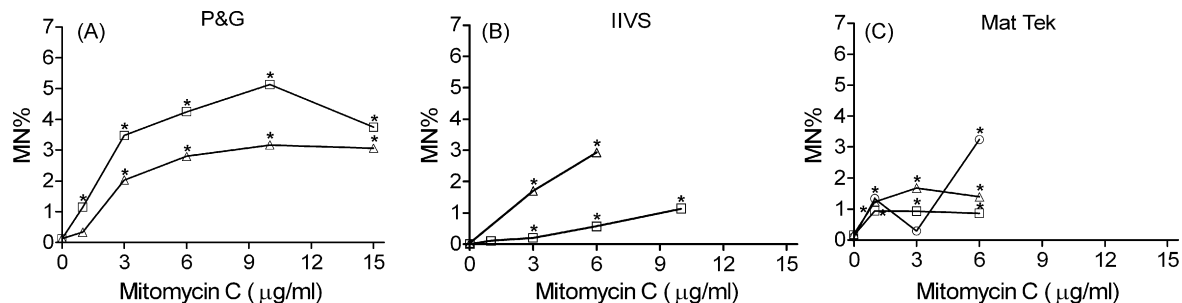


Fig. 2. Mitomycin C-induced micronuclei in tissues from three different donors: (A) for P&G, (B) for IIVS and (C) for MatTek. Triangles for donor 254 tissues, squares for donor 4F1188 tissues, and circles for donor 219 tissues. Asterisks (*) indicate $p < 0.05$ for micronucleus induction.

Table 5
Summary of vinblastine sulfate results using tissue constructed from three different donors tested in three different laboratories.

Compound	Donor	Concentration ($\mu\text{g/ml}$)	Applied on EpiDerm ($\mu\text{g/cm}^2$)	Average binucleation% \pm SD (% decrease compared to solvent control)			% Micronucleated binucleated cells		
				P&G ¹	IIVS ^a	MatTek ^b	P&G	IIVS	MatTek
Vinblastine sulfate	254	0	0	34.7 \pm 5 (0)	30.1 \pm 3.8 (0)	56.1 \pm 4.3 (0)	0.2	0.05	0.06
		0.03	0.001	41 \pm 5.1 (–18.1)	44.7 \pm 0.6 (–44.4)		0.15	0.05	
		0.1	0.003	37.9 \pm 6.8 (–9.3)	41.2 \pm 5.7 (–33.3)	57.7 \pm 3.3 (–2.8)	0.36	1.5	0.5**
		0.3	0.009	26.7 \pm 7.9 (23.1)	32.2 \pm 7.1 (–4)	43.7 \pm 2 (22.1)	0.84**	1.3**	0.42**
		0.6	0.019	9 \pm 4.5 (74)	15.1 \pm 6.9 (51.3)	33.7 \pm 13.7 (40)	3.39**	3.5**	0.36*
		1	0.031	6.3 \pm 3 (81.8)	7.8 \pm 1.1 (74.8)				
	4F1188	0	0	51.4 \pm 1.8 (0)		55.9 \pm 8 (0)	0		0.16
		0.1	0.003	43.2 \pm 4.1 (16)			0.23**		
		0.3	0.009	37.9 \pm 3.1 (26.2)		61.1 \pm 11.2 (–9.3)	0.17*		0.19
		0.4	0.013			61.3 \pm 3.4 (–9.8)			0.43*
		0.6	0.019	24.2 \pm 7.3 (52.9)		41.7 \pm 12.9 (25.4)	2.07**		0.58**
		1	0.031	18.4 \pm 5.5 (64.2)			2.3**		
	219	0	0			58 \pm 8.4 (0)			0.1
		0.3	0.009			50 \pm 6.7 (13.8)			0
		0.6	0.019			52.3 \pm 3.3 (9.9)			0.18
		0.8	0.025			45.8 \pm 4.1 (21)			0.59**

^a Ethanol was used as solvent for P&G and IIVS studies except for the control 4F1188 tissues in the P&G study where acetone was used as solvent.
^b Acetone was used as solvent for MatTek studies.
* $p < 0.05$.
** $p < 0.01$.

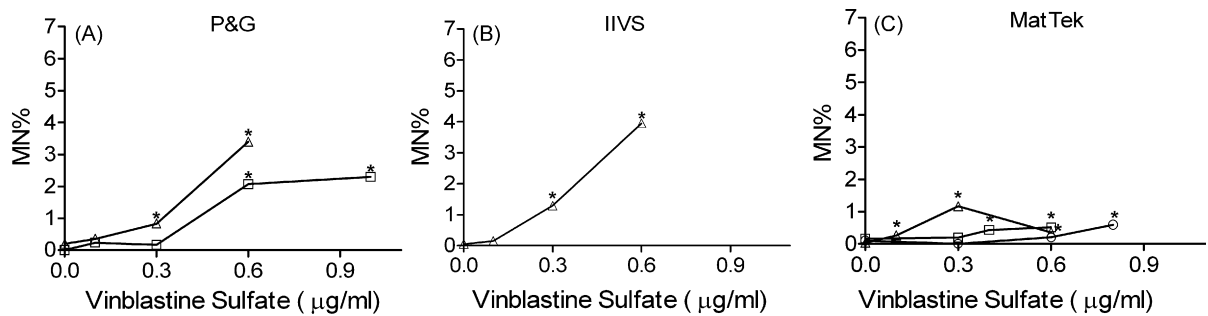


Fig. 3. Vinblastine sulfate-induced micronuclei in tissues from three different donors: (A) for P&G, (B) for IIVS and (C) for MatTek. Triangles for donor 254 tissues, squares for donor 4F1188 tissues, and circles for donor 219 tissues. Asterisks (*) indicate $p < 0.05$ for micronucleus induction.

Table 6
Summary of methyl methanesulfonate results using tissue constructed from four different donors tested in three different laboratories.

Compound	Donor	Concentration ($\mu\text{g/ml}$)	Applied on EpiDerm ($\mu\text{g/cm}^2$)	Average binucleation% \pm SD (% decrease compared to solvent control)			% Micronucleated binucleated cells ¹		
				P&G	IIVS	MatTek	P&G	IIVS	MatTek
Methyl methanesulfonate	254	0	0	35.1 \pm 1.6 (0)	42.4 \pm 4.9 (0)	48.6 \pm 1.8 (0)	0.07	0.07	0
		600	18.75	33.1 \pm 2.1 (5.6)	49.8 \pm 5.9 (–17.5)		0.43**	0.34*	
		800	25	26.6 \pm 5.7 (24.2)	41.1 \pm 2.7 (3.1)	42.6 \pm 11.1 (12.5)	0.43**	0.43**	0.2
		1000	31.25	11.7 \pm 3.1 (66.8)	24.5 \pm 11.1 (42.1)	29.2 \pm 5 (40)	0.72**	0.65**	0.68**
		1200	37.5	10.2 \pm 3.6 (71)	13.4 \pm 5.7 (68.3)	14.5 \pm 7.4 (70.3)	0.60**		0.59**
		926	0	54.1 \pm 2.2 (0)			0.03		
	926	0	0	54.1 \pm 2.2 (0)			0.03		
		600	18.75	42.9 \pm 10.3 (21.8)			0.22*		
		800	25	31.7 \pm 7.1 (41.3)			0.4**		
		1000	31.25	17.9 \pm 4.4 (67)			0.6**		
		1200	37.5	7.8 \pm 3.1 (85.6)					
	4F1188	0	0			55.9 \pm 8 (0)			0.16
		600	18.75			70 \pm 4.4 (–24.9)			0.33
		800	25			44.3 \pm 6.2 (20.6)			0.26
		1000	31.25			34.3 \pm 6 (38.7)			0.44*
	219	0	0			58 \pm 8.4 (0)			0.1
		600	18.75			55 \pm 5.1 (5.1)			0.13
		800	25			63.8 \pm 3.9 (–10.1)			0.21
		1000	31.25			66.2 \pm 6.5 (–14.1)			0.47*

* $p < 0.05$.
** $p < 0.01$.

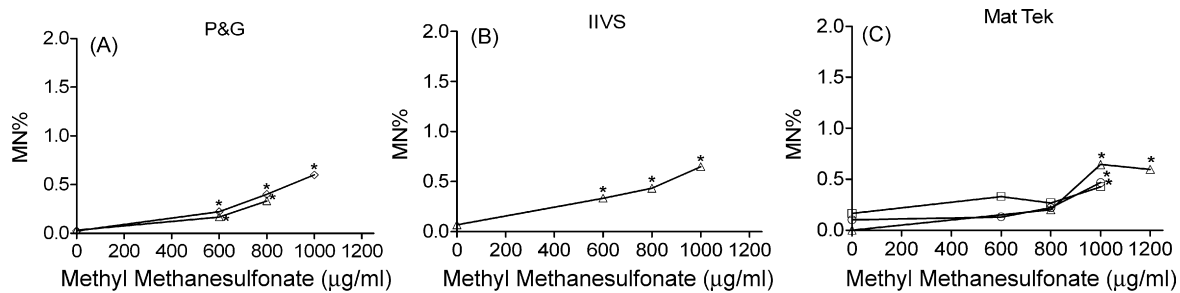


Fig. 4. Methyl methanesulfonate-induced micronuclei in tissues from four different donors: (A) for P&G, (B) for IIVS and (C) for MatTek. Triangles for donor 254 tissues, squares for donor 4F1188 tissues, open or closed circles for donor 219 tissues, vertical rectangles for donor 926 tissues. Asterisks (*) indicate $p < 0.05$ for micronucleus induction.

solvent. A statistically significant, dose dependent increase in MN was observed in the studies conducted at MatTek, but the frequency of VB induced MN appeared lower than that observed at IIVS and P&G, possibly due to different solvents used. Despite the differences in solvents, all three donor tissues tested by MatTek provided consistent toxicity profiles and similar statistically significant levels of MN induction.

3.3.3. Methyl methanesulfonate

We evaluated MMS MN induction in three laboratories using tissues derived from four different donor tissues (254, 926, 4F1188 and 219). As shown in Table 6 and Fig. 4, statistically significant increases in %MN cells were obtained in all laboratories, though the response was lower than for the other two model genotoxins MMC and VB. At 1000 $\mu\text{g/ml}$, MMS induced a statistically significant MN induction in all four donor tissues.

3.3.4. 4-nitrophenol

When tested at concentrations that induced up to 70% toxicity, 4-NP did not induce statistically significant increases of MN level in any experiments with any donor tissues (Table 7 and Fig. 5). A statistically significant increase in MN was observed for the positive control MMC at 3 or 6 $\mu\text{g/ml}$ MMC in all experiments.

3.3.5. Trichloroethylene

TCE was tested in donor 254 tissues by all three laboratories and donor 4F1188 tissues by P&G. As summarized in Table 8 and Fig. 6A,

in the P&G study, 500 mg/ml TCE increased the % relative BN by 60% in the study using donor 254 tissues, but did not affect % relative BN in donor 4F1188 tissues. In contrast, as shown in Fig. 6B, the relative cell yield per tissue decreased over 90% in both experiments using either donor 254 tissues or donor 4F1188 tissues. This indicates that cytotoxicity estimated by the decrease of relative cell yield is a more sensitive measurement for determining the maximum dose for MN analysis for TCE. Based on this, 400 mg/ml TCE was considered the maximum dose for MN analysis to avoid MN analysis for tissues with greater than 70% toxicity. As shown in Table 8 for P&G studies, there were no statistically significant increases of MN frequency at this toxicity level. A statistically significant increase in MN was observed for the positive control MMC or VB in all experiments.

More accurate cell count procedures were incorporated only in the later stage of this project as described in [4], so toxicity of TCE was measured by % relative BN for studies conducted by IIVS and MatTek. TCE did not induce appreciable toxicity as measured by % relative BN in agreement with P&G's studies, however it can be expected that the cell yield was greatly reduced at the high concentrations evaluated by IIVS and MatTek. Even at high concentrations, there were no statistically significant increases in MNBN up to 540 mg/ml IIVS) or 600 mg/ml (MatTek).

3.3.6. 2-Ethyl-1,3-hexanediol

A preliminary study at P&G with EHD ranging from 50 to 75 mg/ml resulted in over 70% cytotoxicity as measured by relative

Table 7

Summary of 4-nitrophenol results using tissue constructed from three different donors tested in three different laboratories.

Compound	Donor	Concentration ($\mu\text{g/ml}$)	Applied on EpiDerm™ (mg/cm^2)	Average binucleation% \pm SD (% compared to decrease control)			% Micronucleated binucleated cells		
				P&G	IIVS	MatTek	P&G	IIVS	MatTek
4-Nitrophenol	254	0	0	48.2 \pm 2.6 (0)	53 \pm 6.7 (0)	37.5 \pm 2 (0)	0	0.07	0
		0.5	0.02	41.1 \pm 10.8 (14.7)			0.06		
		1	0.03	46.3 \pm 5.0 (4)	59 \pm 2.5 (–11.3)	36.5 \pm 0.9 (2.1)	0.05	0.03	0.15
		2	0.06	39.5 \pm 10.0 (18.1)	46.8 \pm 4.1 (11.6)	28.1 \pm 2.7 (24.7)	0.08	0.07	0.2
		3	0.09	25.3 \pm 5.9 (47.6)	31.8 \pm 2.6 (39.9)		0.03	0.03	
		4	0.13	15.9 \pm 3.9 (67.1)	18.6 \pm 8.3 (65)		0.04	0	
		MMC 3 $\mu\text{g/ml}$	0.09 $\mu\text{g/cm}^2$	36 \pm 2.7 (25.3)	45.5 \pm 2 (14.2)	25.8 \pm 3.3 (30.9)	1.55**	0.86**	0.74**
	4F1188	0	0	51.0 \pm 6.2 (0)		56.1 \pm 5 (0)	0	0	0.05
		0.5	0.03	50.0 \pm 6.5 (1.9)			0.05		
		1	0.06	56.6 \pm 9.4 (–10.9)		62.5 \pm 4.8 (–10.5)	0.025		0.15
		2	0.09	40.3 \pm 17.2 (20.9)		48.1 \pm 5.4 (14.2)	0		0.05
		3	0.13	19.5 \pm 10.4 (61.9)		55 \pm 7.5 (2.1)	0		0.2
		4	0.00	16.8 \pm 11.6 (67.1)			0.07		
		MMC 3 $\mu\text{g/ml}$	0.09 $\mu\text{g/cm}^2$	41.8 \pm 4.1 (11.5)		56.6 \pm 2.2 (0.7)	0.95**		0.35*
	219	0	0			58 \pm 8.4 (0)			0.1
		1	0.06			65 \pm 6.3 (–11.9)			0.03
		2	0.09			68.3 \pm 1.5 (–17.7)			0.13
		3	0.13			51.2 \pm 2.1 (11.7)			0
		MMC 6 $\mu\text{g/ml}$	0.19 $\mu\text{g/cm}^2$			43.7 \pm 4.1 (24.6)			3.2**

* $p < 0.05$.

** $p < 0.01$.

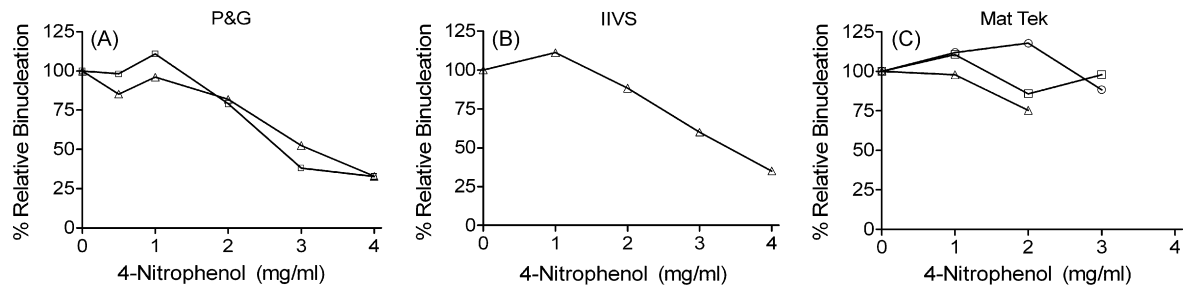


Fig. 5. 4-Nitrophenol-induced cytotoxicity in tissues from three different donors: (A) for P&G, (B) for IIVS and (C) MatTek. Triangles for donor 254 tissues, squares for donor 4F1188 tissues and circles for donor 219 tissues.

Table 8
Summary of trichloroethylene results using tissue constructs from two different donors in three different laboratories.

Compound	Donor	Concentration (mg/ml)	Applied on EpiDerm™ (mg/cm²)	Average binucleation% ± SD (% decrease compared to control)			% Micronucleated binucleated cells		
				P&G	IIVS	MatTek	P&G	IIVS	MatTek
Trichloroethylene	254	0	0	25.1 ± 2.2 (0)	53 ± 6.7 (0)	51.3 ± 9.2 (0)	0.12	0.07	0
		50	1.56	31.2 ± 2.3 (–24.4)			0.13		
		100	3.13	29.1 ± 1.0 (–15.9)			0.3		
		250	7.81	34.9 ± 3.6 (–38.9)			0.27		
		400	12.50			43.4 ± 10.8 (15.4)			0
		500	15.63	41 ± 4.2 (–63.5)	45.5 ± 6.5 (14.2)	53 ± 2.5 (–3.4)		0.05	0.07
		520	16.25		49.9 ± 5.9 (5.8)			0.07	
		540	16.88		41.1 ± 16 (22.5)			0	
		600	18.75			53.6 ± 6.6 (4.6)			0.05
	4F1188	MMC 3 µg/ml	0.09 µg/cm²	24.4 ± 1.6 (2.7)	45.5 ± 2 (14.2)	34.2 ± 5.9 (33.2)	2.03**	0.86**	0.3**
		0	0	51.4 ± 1.8 (0)			0		
		100	3.13	57.1 ± 7.7 (–11.2)			0.03		
		300	9.38	58.2 ± 3.0 (–13.2)			0		
		350	10.94	54.9 ± 3.8 (–6.7)			0.07		
		400	12.50	47.9 ± 7.5 (6.9)			0.03		
		450	14.06	50.7 ± 5.4 (1.4)					
		500	15.63	51 ± 14.1 (0.8)					
		VB 0.6 µg/ml	0.02 µg/cm²	24.2 ± 7.3 (52.8)			2.07**		

** *p* < 0.01.

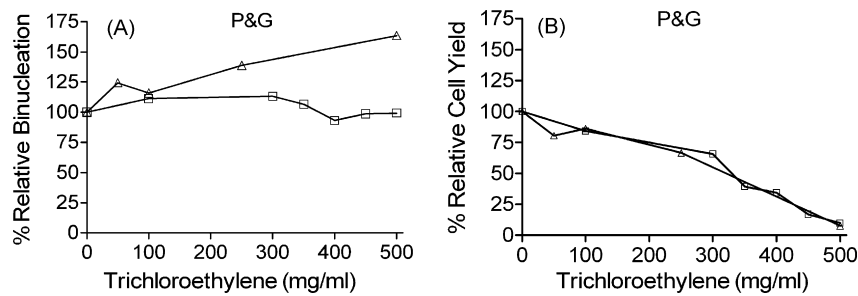


Fig. 6. Trichloroethylene-induced cytotoxicity for P&G studies. Triangles for donor 254 tissues and squares for donor 4F1188 tissues.

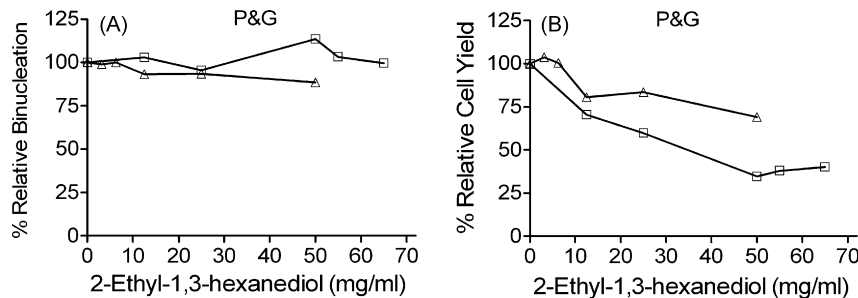


Fig. 7. 2-Ethyl-1,3-hexanediol-induced cytotoxicity for P&G studies. Triangles for donor 254 tissues and squares for donor 4F1188 tissues.

Table 9

Summary of 2-ethyl-1,3-hexanediol results using tissue constructs from two different donors in P&G and IIVS.

Compound	Donor	Concentration (mg/ml)	Applied on EpiDerm™ (mg/cm ²)	Average Binucleation% ± SD (% decrease compared to solvent control)		% Micronucleated binucleated cells	
				P&G	IIVS	P&G	IIVS
2-Ethyl-1,3-hexanediol	254	0	0	55.1 ± 8.9 (0)	53 ± 6.7 (0)	0.025	0.06
		3.125	0.10	54.5 ± 4.4 (1.1)		0.025	
		6.25	0.20	55.2 ± 2.1 (−0.1)		0	
		12.5	0.39	51.2 ± 10.0 (6.8)		0.025	
		25	0.78	51.6 ± 4.1 (6.4)		0.025	
		30	0.94				
		40	1.25				
		50	1.56	48.7 ± 8.7 (11.5)	42.9 ± 3.7 (19)	0.125	0
		60	1.88		30.5 ± 7.7 (42.6)	0	0.08
		70	2.19		14.9 ± 3.2 (71.9)		
		MMC 3 µg/ml	0.09 µg/cm ²	48.7 ± 4.8 (9.3)	45.5 ± 2 (14.2)	1.02**	0.86**
	4F1188	0	0	66.1 ± 0.8 (0)		0.050	
		12.5	0.39	68 ± 7.9 (−2.9)		0.107	
		25	0.78	63.1 ± 7.8 (4.5)		0.000	
		50	1.56	75 ± 5.1 (−13.5)		0.062	
		55	1.72	68.3 ± 9.8 (−3.3)			
		65	2.03	65.8 ± 7.4 (0.5)		0.050	
		MMC 3 µg/ml	0.09 µg/cm ²	59.4 ± 8.2 (10.1)		1.2**	

** $p < 0.01$.**Table 10**

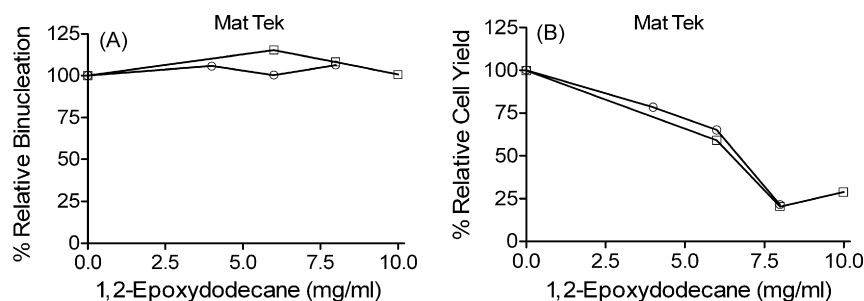
Summary of 1,2-epoxydodecane results using tissue constructs from three different donors in IIVS and MatTek.

Compound	Donor	Concentration (mg/ml)	Applied on EpiDerm™ (mg/cm ²)	Average binucleation% ± SD (% decrease compared to solvent control)		% Micronucleated binucleated cells	
				IIVS	MatTek	IIVS	MatTek
1,2-Epoxydodecane	254	0	0	53 ± 6.7 (0)	43.8 ± 4.1 (0)	0.07	0
		2	0.06	55.8 ± 3.1 (−5.3)		0.1	0
		4	0.13	47.2 ± 1.9 (11)	46.1 ± 2.9 (−4.6)	0.05	0
		6	0.19	53.4 ± 0.9 (0.7)	41.5 ± 10.8 (6)	0	0
		7	0.22	48.3 ± 2.8 (9)		0	
		8	0.25		41.2 ± 0.7 (6.6)		0.1
		MMC 3 µg/ml	0.09 µg/cm ²	45.5 ± 2 (14.2)	35.6 ± 4.9 (18.7)	0.86**	0.34*
	4F1188	0	0		56.1 ± 5 (0)		0.05
		6	0.19		64.7 ± 0.3 (−15.3)		0.05
		8	0.25		60.7 ± 2.1 (−8.1)		0.2
		10	0.31		56.5 ± 13.6 (0.7)		0.25
		MMC 3 µg/ml	0.09 µg/cm ²		56.6 ± 2.2 (0.7)		0.35*
	219	0	0		58 ± 8.4 (0)		0.1
		4	0.13		61.2 ± 0.5 (5.6)		0.05
		6	0.19		58.1 ± 10 (0.3)		0.03
		8	0.25		61.7 ± 3.5 (6.4)		0.07
		MMC 6 µg/ml	0.19 µg/cm ²		43.7 ± 4.1 (24.6)		3.2**

* $p < 0.05$.** $p < 0.01$.

cell yield (data not shown) for all the doses tested. As summarized in Table 9, in P&G studies, no reductions in the BN level (Fig. 7A) were observed when EHD was tested up to 50 mg/ml (donor 254) or 65 mg/ml (donor 4F1188), but cell yield decreased by 50% or more (Fig. 7B). No statistically significant increases in MN frequency after

topical exposure to EHD were observed. As summarized in Table 9, in the IIVS study, topical exposure to 60 and 70 mg/ml EHD caused slightly less than 50% and slightly over 70% toxicity respectively, as measured by relative reduction in the %BN cells. Tissues exposed to 70 mg/ml resulted in slides that were not analyzable due to insuf-

**Fig. 8.** 1,2-epoxydodecane-induced cytotoxicity for MatTek studies. Squares for donor 4F1188 tissues and circles for donor 219 tissues.

ficient cell recovery. In tissues treated with up to 60 mg/ml EHD, there were no increases in MN frequency. A statistically significant increase in MN was observed for the positive control MMC in all experiments.

3.3.7. 1,2-epoxydodecane

IIVS and MatTek evaluated EDD in three different donor tissues (Table 10). Although no reduction in BN level (Fig. 8A) was observed at exposures up to 10 mg/ml EDD, close to 80% cytotoxicity as measured by reduction in cell yield (Fig. 8B) was observed in two MatTek studies performed with donor 4F1188 and donor 219 tissues. In all four studies, there were no dose dependent, statistically significant changes in %MNB after topical exposure to EDD even up to 80% toxicity. A statistically significant increase in MN was observed for the positive control MMC in all experiments.

4. Conclusions

These studies report the intralaboratory and interlaboratory reproducibility across three laboratories of the RSMN MN assay in the EpiDerm™ model. The seven chemicals (genotoxicity and carcinogenicity reviewed in Mun et al. [4]) evaluated in these studies included model genotoxins that function via a variety of mechanisms (e.g. crosslinking agent-MMC, alkylating agent-MMS, aneuploidy inducing agent-VB) and established the ability of this model to respond to different types of damage. Importantly, the studies with the dermal non-carcinogens (4-NP, TCE, EHD, EDD) provide initial data on the specificity of the model which has been a problem area for existing *in vitro* genotoxicity models. All of these chemicals were positive in one or more *in vitro* genotoxicity assays except EDD which has only been evaluated *in vitro* in the Ames assay which was negative (see table in Mun [4]). Due to the 3D nature of EpiDerm™ and the presence of a barrier function, we anticipate that it will provide a more biologically relevant system for dermally applied cosmetics or other chemicals than current *in vitro* genotoxicity assays. Results presented herein for the 4 dermal non-carcinogens lend support to this hypothesis.

Comparable results were obtained in the RSMN assay in the EpiDerm™ model prepared with cells isolated from different tissue donors. Overall, the BN level in negative control tissues from the four different donors (254, 4F1188, 219 and 926) were comparable, and the results with seven test chemicals were comparable. These results are important since the cells used to produce the EpiDerm™ tissue model are primary (non-immortalized) cells and hence have a finite lifetime in culture. As such, different donor cells will be used to construct these models over time. The long term utility and likelihood of regulatory acceptance of assays using such models is dependent on demonstration that the model performs appropriately when prepared using cells from a variety of donors.

Our data demonstrated that for some chemicals, cell yield, as opposed to %BN, appears to be a more sensitive indicator for determining cytotoxicity as evidenced by results with TCE, EDH, and EDD. Decreases in cell yield may be due to interference of the air–liquid interface of the model after topical application of these compounds. We noticed the persistence of a thin layer of liquid film on the top of the tissue surface when TCE, EHD and EDD were tested at high concentrations. Since the air–liquid interface is key to achieving differentiation in these 3D tissue models (<http://www.mattek.com>), disruption of this interface is expected to impact the overall balance between proliferation and differentiation of these EpiDerm™ constructs, and may result in toxicity, the case for TCE, EHD and EDD which resulted in decreased cell counts. A similar observation was reported by Curren et al. [1], where tissues treated with 100 µl of saline caused a 55% reduction in BN level, compared to tissues treated by 10 µl of saline or 10 µl acetone that had no effect on BN level. These observations may point out a practical limit to the solvents and top concentrations of test substances that can be used in this assay.

Overall, these results provide further evidence that the RSMN assay using the EpiDerm™ human skin model is a promising new *in vitro* method for evaluating dermally applied chemicals. The results generated in our three laboratories, provide a basis for more robust validation studies with more laboratories and more test articles as is occurring in a COLIPA sponsored project (<http://www.colipa.com>). We encourage other laboratories to apply these methods to expedite the pace of the development of this promising new non-animal assay to bring us closer to the goal of eliminating laboratory animal usage for cosmetic ingredients.

Conflict of interest statement

Three co-authors (Yulia Kaluzhny, Mitchell Klausner and Viktor Karetsky) of this manuscript are employed by MatTek Corporation, the manufacturer of the reconstructed human tissue model used in the experiments.

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