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The 3D human reconstructed skin micronucleus assay (RSMN) using the EpiDerm™ tissue: Validation and application to the safety assessment of cosmetics ingredients

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Cosmetics Europe
the personal care association

Introduction

Regulatory restrictions on animal use have increased the reliance of risk assessors and regulators on *in vitro* test systems. *In vitro* assays are usually based on mammalian cell culture systems using 'immortal' cells with compromised cell functions. Such 2-dimensional static cell culture systems are artificial and far removed from the *in vivo* state, while 3D tissue constructs allow for more natural cell-cell and cell-matrix interactions and show '*in vivo like*' behavior. Ideally, tissue-based assays could replace the animal studies as follow-up tools to verify results from standard *in vitro* assays. The RSMN assay combines the EpiDerm™ 3D reconstructed skin (RS) model with the micronucleus (MN) assay to provide a more realistic model for evaluating the genotoxic potential of dermally applied chemicals or products, such as cosmetics. This assay is expected to be used as a follow-up for positive results from the standard *in vitro* genotoxicity battery[1]. Cosmetics Europe has funded the establishment and evaluation of the RSMN assay and shown it to have good transferability, inter- and intra-laboratory reproducibility in validation studies[2,3]. In Phase 3, the predictive capacity of the assay was explored and the sensitivity observed with the standard 48h treatment protocol was insufficient (65%) which led to assay modifications (extension of the treatment from 48h to 72h). Bridging studies with 12 coded chemicals were performed to evaluate the performance of the modified protocol which included a 72h treatment as verification of negative or equivocal results in the initial 48h treatment.

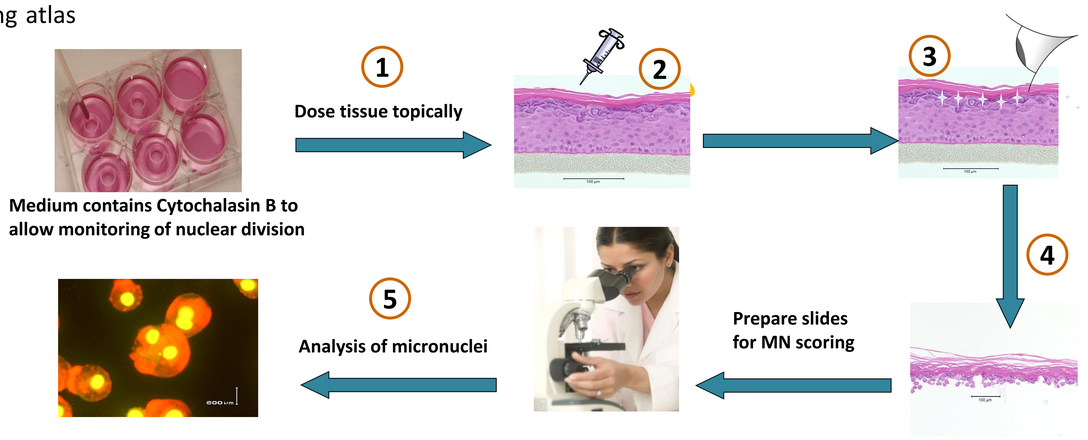
Methods

A detailed protocol for the 3D skin MN assay was published, together with a harmonized scoring atlas for micronuclei[4]. An overview of the method and the modified criteria is shown below.

1. EpiDerm™ models are treated topically with test compound.
2. Dose at 24h intervals (48h or 72h total)
3. Precipitation at the beginning and the end of the treatment period is noted.
4. Keratinocytes are released by trypsinization
5. Micronuclei in binucleated cells are counted by visual scoring.

Additional criteria were applied:

- The lowest precipitating concentration was the highest dose for the evaluation of micronuclei
- A negative outcome in the first 48h experiment was verified by additional 72h experiments. If the results were positive at 72h, the overall call was positive



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Results

The validation data are summarized in Table 1. When chemicals were tested in the updated protocol, and in the same laboratory, the data from these 'bridging studies' were accepted as the final call. Since very good inter-lab reproducibility was obtained in previous studies, some chemicals were tested by a single laboratory only. Overall, the data demonstrate and excellent **overall specificity (87%)** in the RSMN assay with only few mispredictions: diclofenac (3/3 labs), phenanthrene (1/4), resorcinol (1/2) and curcumin (1/1 lab - also positive in all other *in vitro* assays). Considering sensitivity, there were 6 true positive chemicals that were negative initially, using a 48h dosing regimen, but were positive when tested in a 72h dosing regimen. The inclusion of a 72h dosing regimen increased the **sensitivity to 80%**. Two out of the three chemicals that were missed by the 72h regimens, totally or partially (2-AAF and $CdCl_2$), have also been tested in the process of validation of the 3D skin Comet assay and were found genotoxic in this assay. This suggest that the calculated sensitivity presents a conservative estimate of the sensitivity of tissue-based genotoxicity assays since both 2-AAF and $CdCl_2$ are Ames positive compounds which would have been be picked up if tested in an endpoint-driven approach, increasing the **sensitivity to 92%.**

Table 1. Summary table of all interpretations relevant for the predictive capacity assessment. Validation compounds were classified as negative (Table 1A), true positive (Table 1B) or false positive (Table 1C). The "correct result" column shows in brackets the number of labs that correctly identified chemical and the number of labs that tested it (including the 72h repeat experiments)

1 (A) 'True negatives'

Test substance	Lab 1	Lab 2	Lab 3	Lab 4	Result
Ampicillin sodium salt	-	-	Neg	-	1/1
Beclomethasone dipropionate	Neg	-	-	-	1/1
Cyclohexanone	Neg	-	Neg	Neg	3/3
Diclofenac [a]	Pos	-	Pos	Pos	0/3
d-Limonene	Neg*	Neg*	Neg	-	3/3
Mannitol	Neg	-	-	Neg	2/2
n-Butyl chloride	Neg	Neg*	Neg*	Neg	4/4
Nifedipine	-	-	Neg	-	1/1
Phenanthrene[b]	Neg	Neg	Pos	Neg	3/4
Tolbutamide	Neg	Neg	Neg	-	3/3

	Final call negative (correct call)
	Final call a false positive
-	Not tested

1 (B) 'True positives'

Test substance	Lab 1	Lab 2	Lab 3	Lab 4	Result
2-Acetyl animofluorene	Neg*	Neg*	-	Neg	0/3
2,3-Dibromo-1- propanol	-	-	Pos	-	1/1
2,4-diaminotoluene	Neg*	-	Pos	Neg	1/3
4-Vinyl-1-cyclohexene diepoxide	Pos	Pos*	Pos*	-	3/3
Ethylnitrosourea	Pos	-	Pos	Pos	3/3
Etoposide	Pos	-	Pos		2/2
Mitomycin C	Pos	-	Pos	Pos	3/3
Methyl methanesulfonate	Pos	-	-	-	1/1
Colchicine	-	Pos	Pos	-	2/2
Cyclopenta(c,d)pyrene	Pos*	Neg	-	-	1/2
Ethyl methansulfonate	-	Pos	Pos	-	2/2
5-fluorouracil	Neg*	-	Pos*	-	1/2
Taxol (Paclitaxel)	-	Pos	-	-	1/1
Potassium bromate	Pos	Pos*	Pos	-	3/3
Cytosine arabinoside	Pos*	-	-	-	1/1
Diethylstilbestriol	Pos*	-	-	-	1/1
Cadmium chloride	Neg*	+/-*	+/-*	-	1/3

	Final call a false negative
	Final call inconclusive or equivocal
	Final call positive (correct call)

1 (C) 'False' or 'irrelevant' positives

Test substance	Lab 1	Lab 2	Lab 3	Lab 4	Result
1-Nitronapthalene	-	-	Neg	-	1/1
2,4-Dichlorophenol	-	-	Neg	Neg	2/2
2,6-Diaminotoluene	-	-	-	Neg	1/1
8-Hydroxyquinoline	-	-	-	Neg	1/1
Curcumin	Pos	-	-	-	0/1
Ethionamide	Neg*	Neg*	Neg	-	3/3
Nitrofurantoin	Neg	-	-	-	1/1
Phenol	-	-	-	Neg	1/1
p-Nitrophenol	Neg	Neg*	Neg*	Neg	4/4
Propyl gallate	Neg	-	-	-	1/1
Resorcinol	Neg*	-	Pos	-	1/2

	Final call negative (correct call)
	Final call a false positive
-	Not tested

Conclusion

- **An international validation study with 38 coded chemicals shows a high sensitivity (80%) and specificity (87%) for the prediction of *in vivo* genotoxicity outcomes**
- **Two of the compounds missed are Ames positive and would be picked up by the 3D skin Comet assay, which increases the sensitivity to 92%**
- **The data supports the use of the human 3D skin-based genotoxicity assays for follow-up of unfavourable results from standard *in vitro* assays (e.g., Ames, micronucleus) and therefore is a direct replacement of *in vivo* follow-up testing**

References: 1-Pfuhler *et al*. Reg Pharm Tox 2010; 57(2-3):315-324; 2-Hu *et al*. Mutat Res. 2009, 673(2):92-9; 3-Aardema *et al*. Mutat Res. 2010, 701(2):123-31; 4-Dahl *et al*. Mutat Res. 2011 28;720(1-2):42-52..
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