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RSMN (Reconstructed Skin Micronucleus Assay): Update on the ongoing validation

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Introduction

3D reconstructed skin tissues provide a more realistic model for dermally applied chemicals/products, such as cosmetics, and are expected to be used to follow-up on positive results from the *in vitro* genotoxicity battery[1]. Phase 1 and 2 of the RSMN validation using EpiDerm™ tissues showed good transferability, inter- and intra-laboratory reproducibility[2,3]. In Phase 3, the number of chemicals was extended to 29 (Table 1). Results demonstrated excellent specificity and good within-laboratory reproducibility (Table 2 and 3), while sensitivity needs further investigation.

Table 2: Statistical analysis of assay performance

Parameter	Weighted
Specificity	18.5/21 = **88.1%**
Sensitivity	5/8 = **62.5%**
Concordance	23.5/29 = **81%**

Table 3: Statistical analysis of within-laboratory reproducibility of assay

Lab 1	Lab 2	Lab 3
85.7% (12/14)	**80.0% (12/15)**	**93.3% (14/15)**

The number of true positives tested so far (8) was considered too few to draw a final conclusion about the sensitivity of this assay. Therefore, an additional 12 compounds identified by external experts are currently being tested, with a focus on genotoxic carcinogens.

This poster presents:

- **An update on the progress of the validation**
- **Data showing the successful transfer of the protocol to a third laboratory (since Lab 2 was no longer able to participate)**

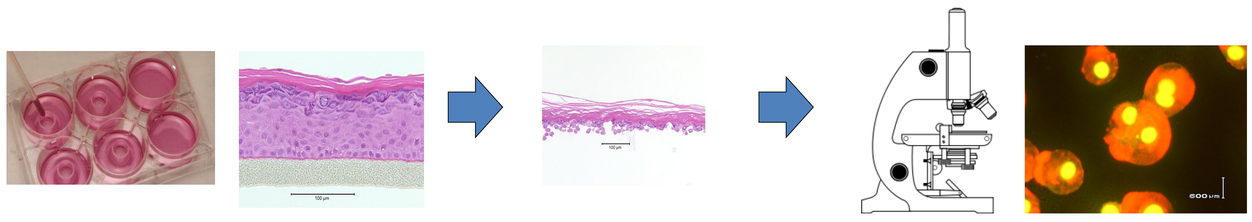
Table 1. Summary table of all interpretations relevant for the predictive capacity assessment. Compounds were classified as negative (N), true positive (TP) or false positive (FP). Results are shown and grey boxes denote a false positive or negative interpretation; bold text denotes an inconclusive overall interpretation.

Test Material	Expected Result	Interpretation at			Overall interpretation
		Lab 1	Lab 2	Lab 3	
Ampicillin sodium salt	N	-	-	negative	negative (1)
Beclomethasone dipropionate	N	negative	-	-	negative (1)
Cyclohexanone	N	negative	negative	negative	negative (3)
Diclofenac	N	negative	positive	-	**negative (1) positive (1)**
d-Limonene	N	-	-	negative	negative (1)
Mannitol	N	negative	negative	-	negative (2)
n-Butyl chloride	N	negative	negative	negative	negative (3)
Nifedipine	N	-	-	negative	negative (1)
Phenanthrene	N	-	negative	positive	**negative (1) positive (1)**
Tolbutamide	N	negative	-	positive	**negative (1) positive (1)**
1-Nitronapthalene	FP	-	-	negative	negative (1)
2,4-Dichlorophenol	FP	-	negative	negative	negative (2)
2,6-Diaminotoluene	FP	-	negative	-	negative (1)
8-Hydroxyquinoline	FP	-	negative	-	negative (1)
Curcumin	FP	positive	-	-	positive (1)
Ethionamide	FP	-	-	negative	negative (1)
Nitrofurantoin	FP	negative	-	-	negative (1)
Phenol	FP	-	negative	-	negative (1)
p-Nitrophenol	FP	negative	negative	-	negative (2)
Propyl gallate	FP	negative	-	-	negative (1)
Resorcinol	FP	-	-	negative	negative (1)
2-Acetylaminofluorene (2-AAF)	TP	-	negative	-	negative (1)
2,3-dibromo-1-propanol	TP	-	-	positive	positive (1)
2,4-Diaminotoluene (2,4-DAT)	TP	-	negative	-	negative (1)
4-Vinyl-1-cyclohexene diepoxide (4-V-1-CHD)	TP	-	negative	-	negative (1)
N-Ethyl-N-nitrosourea (ENU)	TP	positive	positive	positive	positive (3)
Etoposide	TP	positive	-	positive	positive (2)
Mitomycin C (MMC)	TP	positive	positive	positive	positive (3)
Methyl methane-sulfonate (MMS)	TP	positive	-	-	positive (1)

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 is shown below.

- EpiDerm™ models are treated topically with test compound.
- Two doses at 24 h intervals – a total of 48 h incubation
- Precipitation at the start and the end of the treatment period was noted.
- Medium contains Cytochalasin B to allow monitoring of nuclear division
- Keratinocytes are released by trypsinization
- Micronuclei in binucleated cells are counted by visual scoring.



Results

- **The protocol was successfully transferred to new laboratories.** There was a very good comparison of the % MN in EpiDerm™ models treated with acetone or MMC in all three laboratories (Figure 1). Although the % MN in models treated with 3 μg/mL in Lab 4 was lower compared to the other laboratories, there was a similar fold-increase given the lower background vehicle control values in lab 4. A higher dose of MMC (5 μg/mL) resulted rate of MN formation comparable to labs 1 and2 and was therefore included in all subsequent assays. The within laboratory reproducibility in Lab 4 was very good for coded chemicals (Figure 2, independent analysis in progress).

- **All chemicals have now been tested.** Since there was a very good inter-lab reproducibility in the previous studies (Table 1), in this phase, the majority of chemicals (20) were tested by a single laboratory. Five chemicals were tested by two laboratories and four chemicals were tested by all three laboratories. The data to date are summarized in Table 1. The results from the remaining 12 coded chemicals has just become available and a detailed statistical analysis is in progress. Sensitivity (5/8, 63%) is less than hoped for due to false negative outcomes. Follow-up is ongoing for the chemicals that were missed: (1) 2-AAF was negative → needs CYP activation; (2) 4-V-1-CHD, a rat skin carcinogen, was negative → needs CYP activation; (3) 2,4 DAT was positive in another laboratory (Pfuhler et al., in preparation). Notably, 2-AAF and 2,4-DAT were positive in the RS Comet assay, while 4-V-1-CHD has not yet been tested with the 3D skin Comet assay.

- **Initial studies addressing bioactivated genotoxins have started.** In order to address the lack of bioactivation of some of the pro-mutagens, we are currently investigating whether the addition of Aroclor induced rat liver S9 in the medium could increase the sensitivity of the test system. Two previously tested bioactivated genotoxins (benzo[a]pyrene and cyclophosphamide) that gave either a weak response or required higher doses in the RSMN assay were selected for these assays. Initial studies suggest that addition of 2% S9 to the medium below the skin model during the first 4 h of chemical treatment improves the sensitivity of the assay (data not shown). An additional option for bioactivated genotoxins is to increase the exposure to 72 h, as previously reported[5].

Conclusion

- **Overall, the data generated to date support the use of the 3D skin EpiDerm™ model for genotoxicity testing of dermally applied chemicals.**
- **The protocol was successfully transferred to a fourth laboratory, which demonstrated comparable control values to the other 2 laboratories and excellent intra-experimental reproducibility.**
- **This current study on 12 additional chemicals is expected to provide a conclusive estimation of the sensitivity and specificity of the RSMN.**

Figure 1

Reproducibility of the RSMN assay among laboratories. % MN in EpiDerm™ models treated with vehicle (acetone, light coloured bars) and positive (MMC, dark coloured bars with the indicated dose below the axis) controls. Values are means from 4-7 experiments ± SD; * = statistically different from concurrent vehicle control (P<0.05).

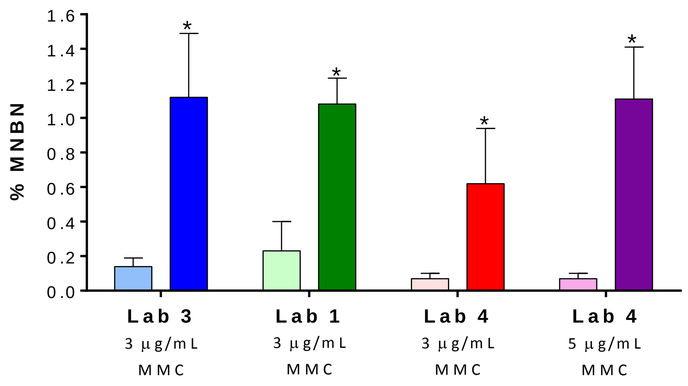
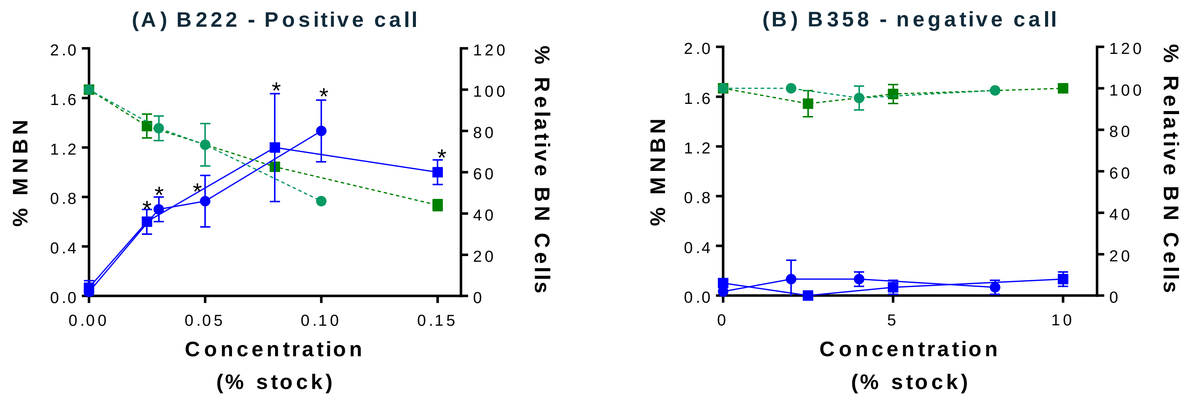


Figure 2

Figure 2. Reproducibility of the RSMN assay within Lab 4. % MN in EpiDerm™ models treated with (A) coded chemical B222 and (B) B358. Circles = Experiment 1, Squares = Experiment 2. Green = % Relative BN cells, blue = %MNBN. Values are mean ± SD, * = statistically different from concurrent vehicle control (P<0.05).



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; 5- Aardema *et al*. Mutat Res. 2013 750(1-2):40-9.

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