Reconstructed 3D Human Skin Micronucleus Assay: Preliminary Pre-Validation Results

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Introduction

The skin is the main route of exposure of many chemicals and cosmetic ingredients; therefore, Cosmetics Europe (formerly COLIPA) has funded and driven projects to establish and evaluate more realistic models for genotoxicity using 3D reconstructed skin (RS) tissues. The aim is to use these to follow-up on positive results from the in vitro genotoxicity battery[1], which has been criticized for its low specificity. The RS model, EpiDerm™, was combined with the micronucleus (MN) assay and the resulting 3D skin MN assay exhibited good intra- and inter-laboratory reproducibility[2], and correctly identified 3 coded chemicals as being either positive or negative[3]. As part of the third phase pre-validation process of this project, we have extended the number of coded chemicals tested to 29. Here, we present the outcome of this testing phase.

Phase 3 testing strategy

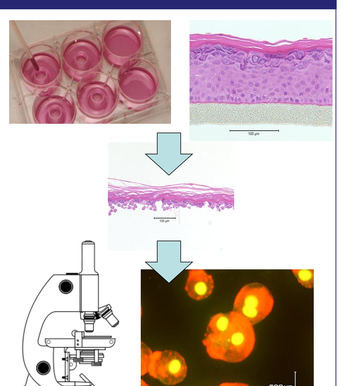
"Efficient" approach (across 3 laboratories):

- At least 2 laboratories tested 10 chemicals (inter-laboratory reproducibility)
- An additional 19 coded chemicals were tested by only one laboratory (expansion of database)
- Chemical classes tested:
  - 8 True positives
  - 11 False positives
  - 10 True negatives
- At least 2 valid experiments per chemical
- All results were sent to ECVAM for decoding and evaluation according to specific predetermined criteria

Method

A detailed protocol for the 3D skin MN assay was published, together with a harmonized scoring atlas for micronuclei[4].

- EpiDerm™ models are treated topically with test compound.
- Two doses – a total of 48 h incubation
- Medium contains Cytochalasin B
- Keratinocytes are released by trypsinization
- Micronuclei in binucleated cells are counted by visual scoring.



Results

The outcome of the testing of 29 coded chemicals using an "efficient approach" (see panel "Phase 3 testing strategy") is shown in Table 1. The intra-laboratory reproducibility was high (Table 2) and there was good overall specificity (Table 3). Moreover, the 3D skin MN assay detected direct acting genotoxins, as well as genotoxins that require metabolism.

Three of the 8 genotoxic chemicals were negative. These were:

- 4-Vinyl-1-cyclohexene diepoxide: A rat skin carcinogen which needs CYP activation, the levels of which are low in human skin[5,6].
- 2-AAF: A weak clastogen that is not easy to detect in a traditional *in vitro* MN test. It is bioactivated by CYP1A2, the activity of which is very low in skin[5].
- 2,4-DAT: Difficult to obtain a positive even in standard *in vitro* genotoxicity assays.

There were 3 compounds expected to be negative but were positive. Of these, 2 were under conditions of severe precipitation. Avoiding this may avoid a positive call.

Conclusions

- There was an excellent specificity (88%), demonstrating that the 3D skin MN assay has a good potential to improve the specificity of *in vitro* genotoxicity assays as a whole.
- Of the 8 carcinogens with a suggested genotoxic mode of action, 5 were correctly predicted. For the 3 that were missed we believe that there is a valid hypothesis available of why they were not picked up by the 3D skin model
- While this indicates good sensitivity, the total number of true positives was considered too low to draw a final conclusion about the sensitivity of this assay. Therefore more coded compounds will be tested in a next project phase with a focus on carcinogens.
- Overall, these data support the use of the 3D skin EpiDerm™ model for genotoxicity testing of dermally applied chemicals.

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
		IIVS	L'Oréal	P&G	
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	TP	-	negative	-	negative (1)
N-Ethyl-N-nitrosourea (ENU)	TP	positive	positive	positive	positive (3)
Etoposide	TP	positive	-	positive	positive (2)
Mitomycin C	TP	positive	positive	positive	positive (3)
Methyl methane-sulfonate (MMS)	TP	positive	-	-	positive (1)

Table 2: 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)**

Table 3: Statistical analysis of assay performance

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

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References

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