Ongoing Validation Studies for the 3D Human Reconstructed Skin Micronucleus and Comet Assays

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Introduction

3D reconstructed skin (RS) models have been combined with the micronucleus (MN) and Comet assays to provide more realistic models for evaluating the genotoxic potential of dermally applied chemicals/products, such as cosmetics. The development of these assays has been encouraged by external groups (IWGT, ECVAM, SCCS), and they are expected to be used as follow-ups for positive results from the standard *in vitro* genotoxicity battery[1]. We present the overall findings of Phase 3 testing of the RSMN assay and the key modifications to the protocol that led to an increased sensitivity. We also present the results of the initial validation phase of the RS Comet assay using Phenion® FT tissues.

RSMN assay

Methods

A detailed protocol for the 3D skin MN assay was published, together with a harmonized scoring atlas for micronuclei[2].

Additional criteria applied:

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

Table 1. Summary table of all interpretations relevant for the predictive capacity assessment. Compounds were classified as negative (Table 1A), false positive (Table 1B) or true 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)

(A) True negatives

Test chemical	Correct result?
Ampicillin sodium salt	Yes (1/1)
Beclomethasone dipropionate	Yes (1/1)
Cyclohexanone	Yes (3/3)
Diclofenac	Yes (1/2)
d-Limonene	Yes (1/1)
Mannitol	Yes (2/2)
n-Butyl chloride	Yes (3/3)
Nifedipine	Yes (1/1)
Phenanthrene	Yes (3/4)
Tolbutamide	Yes (2/3 (3rd 1+ve &1-ve)

(B) False positives

Test chemical	Correct result?
1-Nitronapthalene	Yes (1/1)
2,4-Dichlorophenol	Yes (2/2)
2,6-Diaminotoluene	Yes (1/1)
8-Hydroxyquinoline	Yes (1/1)
Curcumin	No (1/1)
Ethionamide	Yes (1/1)
Nitrofurantoin	Yes (1/1)
Phenol	Yes (1/1)
p-Nitrophenol	Yes (2/2)
Propyl gallate	Yes (1/1)
Resorcinol	Yes (1/1)

(C) True positives

Test chemical	48h Dosing				72h Dosing	Correct result?
	Lab 1	Lab 2	Lab 3	Lab 4		
2-Acetyl animofluorene	-	-	-	negative		No (1/1)*
2,3-Dibromo-1- propanol	-	-	positive	-		Yes (1/1)
2,4-diaminotoluene	negative	-	positive	negative	negative	No (2/3)
4-Vinyl-1-cyclohexene diepoxide	-	-	-	negative	positive	Yes (1/1)
Ethylnitrosourea	positive	-	positive	positive		Yes (3/3)
Etoposide	positive	-	positive			Yes (2/2)
Mitomycin C	positive	-	positive	positive		Yes (3/3)
Methyl methanesulfonate	positive	-	-	-		Yes (1/1)
Colchicine	-	positive	positive		-	Yes (2/2)
Cyclopenta(c,d)pyrene	negative	negative	-		positive	Yes (1/2)
Ethyl methansulfonate	-	-	positive		-	Yes (1/1)
5-fluorouracil	-	-	negative		positive	Yes (1/1)
Taxol (Paclitaxel)	-	positive	-		-	Yes (1/1)
Potassium bromate	-	negative	-		-	Yes (1/1)
Cytosine arabinoside	negative	-	-		positive	Yes (1/1)
Diethylstilbestriol	negative	-	-		positive	Yes (1/1)
Cadmium chloride	negative	-	-		negative	No (1/1)*

Legend: False positive; False negative; -: not tested; *positive in skin Comet

Results of validation

Specificity: Overall **specificity was > 90%** with only 3 false-positive results: diclofenac, tolbutamide and curcumin (also positive in all other *in vitro* assays). Results for phenanthrene (a true negative with precipitating doses) were equivocal in 1 lab but negative in 3 other labs.

Sensitivity: There were 6 true positive chemicals that were negative using a 48h dosing regimen but were positive when tested in a 72h dosing regimen. This included 4-vinyl-1-cyclohexene diepoxide which needs metabolic activation. The inclusion of a 72h dosing regimen increased the **sensitivity to 88%** (15/17 chemicals were correctly identified). Two out of the 3 chemicals that were missed by the 72h regimens (2-AAF and $CdCl_2$) have been tested in the 3D skin Comet assay and were genotoxic in this assay, showing that these Ames positive compounds will be picked up if tested in an endpoint-driven approach.

RS Comet assay

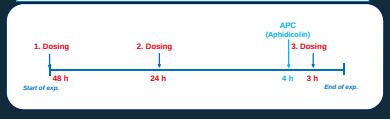
The validation includes testing 30 chemicals, selected by external experts, in an incomplete block-design. The initial testing phase is completed and focused on inter- and intra-laboratory reproducibility using the Phenion® Full-Thickness Skin model.

Methods

Eight coded chemicals were each evaluated in three laboratories using the following dosing regimen:

Phenion® Full Thickness Skin model

Dosing regimen for the 3D Skin Comet assay



The tissues are treated 3 times (48, 24, and 3 h before cell isolation) to allow for the detection of pro-mutagens and to ensure the detection of acute DNA damage. If the outcome of the first experiment was negative, a subsequent experiment included aphidicolin (APC) to increase sensitivity of the assay. APC inhibits the final step of specific DNA-repair processes and induces an accumulation of single strand breaks, which increases the Comet signal.

Table 2. Outcome of experiments by different laboratories. TP = true positive, FP = false positive, TN = true negative, purple = inconclusive, yellow = false negative.

Test chemical	Type	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Mitomycin C (MMC)	TP – Direct – cross-linker	positive	positive		inconclusive	
Cadmium chloride	TP – Direct			positive	negative	positive
N-Ethyl-N-nitrosurea	TP- Direct	positive	positive	positive		
7,12-Dimethylbenz(*a*)anthracene	TP-Bioactivated		positive	positive		positive
Eugenol	FP	negative	negative			negative
Propyl gallate	FP			negative	negative	negative
Cyclohexanamone	TN	negative		negative	negative	
Di-(2-thylhexyl)phthalate)	TN		negative		negative	negative
	Predictivity	**100%**	**100%**	**100%**	**80%**	**100%**

Results of initial validation

The Phenion® FT model was well suited to the comet assay since:

- There was a good overall predictivity of the expected genotoxicity. Four of the labs, correctly identified all 5 chemicals and the fifth correctly identified 80% of the chemicals (Table 2).
- Comets in negative and solvent control tissues were small.
- Results on the solvent and positive control (MMS or B[*a*]P) were comparable among labs.
- There was a good reproducibility within and between labs.

Detection of crosslinkers

The outcome for Mitomycin C (MMC) was inconclusive in Lab 3 but was correctly classified as positive by Lab 1 and 2. MMC is a DNA cross-linker that intercalates between DNA strands, leading to covalent binding. This activity also affects fragmented DNA such that positive Comet signals can be suppressed at higher doses[3]. The suppression of the MMS-induced DNA damage by MMC can also demonstrated using the Phenion® FT model (data not shown). The incorporation of MMS might help to efficiently detect crosslinkers.

Conclusions

RSMN assay:

- **Overall, these data support the use of the RSMN assay using the EpiDerm™ model as a novel in vitro assay for genotoxicity testing of dermally applied chemicals**
- **Final bridging studies are planned to confirm that the 72h dosing regimen improves the sensitivity without compromising the specificity of the RSMN assay**

RS Comet assay:

- **These data support the use of the Comet assay using Phenion® FT tissue since the predictivity for 8 coded chemicals was good in all laboratories.**
- **Testing will be continued with the Phenion® FT model to obtain a complete data set for all 30 chemicals.**

References: 1-Pfuhler *et al.* Reg Pharm Tox 2010; 57(2-3):315-324; 2-Dahl *et al.* Mutat Res. 2011 28;720(1-2):42-52; 3-Pfuhler & Wolf Environ Mol Mutagen. 1996;27(3):196-201

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