In-vitro-only testing strategies for cosmetic ingredients: Utility of newly validated genotoxicity assays using human skin and fertilized hen's eggs

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Abstract

The reconstructed skin micronucleus (RSMN) and the 3D skin Comet are two genotoxicity tests have been recently validated and show very promising results in terms of predictive capacity (sensitivity, specificity). The hen's egg test (HETMN) recently underwent a validation study and also exhibits a very good predictive capacity. These three tests incorporate metabolic and route-of-exposure considerations in relation to the safety assessment of cosmetics. Their high predictive capacity makes them very good candidates to be incorporated in testing strategies and address the issue of generation of a high percentage of misleading positive results by current standard in vitro testing battery. Skin-based assays (Comet and micronucleus) are addressing the dermal route of exposure while the Hen's Egg Micronucleus (HET-MN) better represents an oral exposure pathway. We report here examples of possible testing strategies and their overall performance.

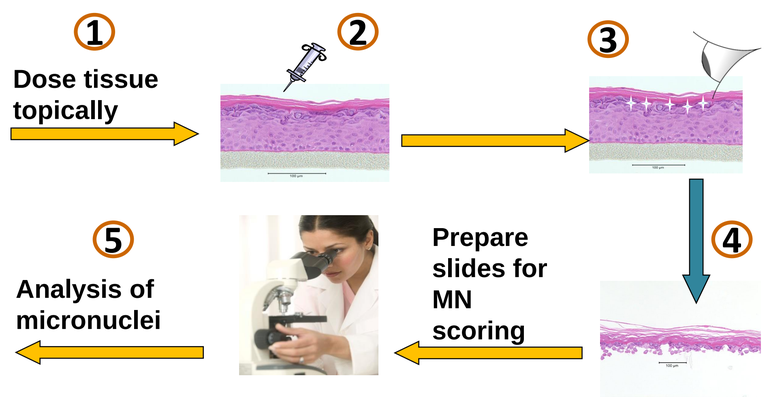
Reconstructed Skin Micro Nucleus (RSMN)

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 **(in bold)** 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**



Parameter	Phase 1-3 testing	+ Bridging studies
Sensitivity	62.5%	80.0%
Specificity	95.2%	87.0%
Overall concordance	86.2%	89.5%

n = 38 chemicals, 17 known actual rodent carcinogens, 21 actual non-rodent carcinogens

- 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%

3D-Skin Comet

Cell separation → **Test Protocol** → **Comet assay procedure**

Phenion® Full-Thickness Skin Model
www.phenion.com

Epidermis → Mincing* (mechanical treatment)
Dermis → Mincing* (mechanical treatment)
→ Single cells re-suspended in low melting agarose
→ Transfer to glass slides
→ Lysis overnight*
→ Unwinding* 20 min, pH 13
→ Electrophoresis* 30 min
→ Neutralization* Ethanol Slides air-dried

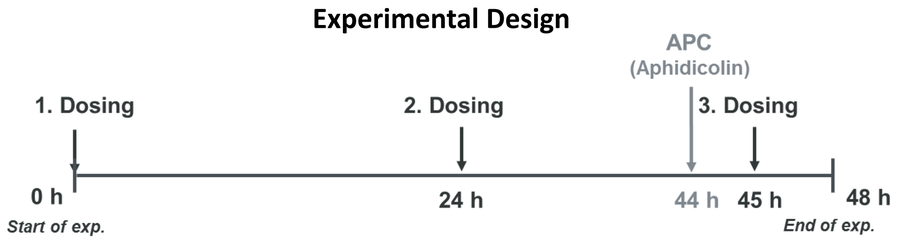
* Buffers according to Tice, 2000

Study design

(A) Solubility study: acetone, 70% ethanol [v/v]
(B) Dose-range finding experiment (max. dose 10% in 25 µl solvent)
(C) At least two valid main experiments

Negative findings are further investigated using aphidicolin (APC): APC - inhibitor of DNA polymerases α + δ, which both possess excision repair function ⇨ APC induces accumulation of DNA strand breaks evolved during DNA repair processes to amplify comet formation.

Experimental Design



- Topical application of tissues for 48 h to allow for up-regulation of phase I xenobiotic metabolism. 3 h treatment allows for detection of DNA damage subject to fast repair.
- Experiment comprises: (a) solvent control (acetone, 70% ethanol [v/v]); (b) positive control (MMS, standard; APC protocol ± BaP); (c) at least three concentrations of test compound. Three tissues used per control/dose group
- Negative findings are further investigated in an APC experiment (APC added 4 h before sampling to cell culture medium).

Parameter	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Overall
No. chemicals	9	14	12	5	4	28
Sensitivity	100	75	80	25	100	**73**
Specificity	100	83	85	100	100	**87**
Accuracy	100	79	83	70	100	**80**

Hen's Egg Test – MicroNucleus (HET-MN)

Test Design

Application on day 8 of chicken egg development

Sampling on day 11

Microscopical analysis of erythrocytes

Study design
(1) Solubility study
(2) Dose range finder. Max. dose 100 mg/egg, i.e., in line with max. dose *in vivo*.
(3) At least 2 main experiments

Experimental design
- Solvent control: aqua DI, isopropyl myristate, 10% DMSO/EtOH
- Positive control: cyclophosphamide (pro-mutagen)
- At least three concentrations of test compound

Read-out parameter
- Evaluation of 6 eggs per dose group with 1000 cells per egg, i.e., 6000 cells per dose/control group
- Micronucleus (MN) frequency in erythrocytes
- Viability of eggs
- Change of PCE/NCE ratio considered not sensitive enough

Advantages of the Assay

HET-MN mirrors systemic availability of compounds
- Absorption
- Distribution via the vessel system
- Metabolism in the developing liver and the yolk sac membrane
- Excretion of metabolite into allantois (bladder equivalent)

- Not an animal experiment
- Clear intrinsic metabolic activity omits the need to add rat liver S9 mix
- Chicken eggs (SPF eggs used for vaccine production) globally available
- So far no difference observed between suppliers/strains

Parameter	Lab 1	Lab 2	Lab 3	Overall
No. of chemicals	14	20	23	32
Sensitivity	57	100	80	88
Specificity	100	100	92	97
Accuracy	79	100	87	92

Data evaluation
(1) Validity check
- e.g. bioavailability check of test compound by modification of MN rate or vitality

(2) Statistical analysis
- Prediction model 1:lab-specific threshold /trend test
- PM 2:Umbrella-Williams-Test (Hothorn *et al.*, 2013)

(3)Consideration of biological relevance

Conclusion

- The RSMN and the 3D-Comet address the dermal route for the first time in *in vitro* genotoxicity testing. All three types of DNA damage, to be considered in regulatory decision-making, can be evaluated.
- 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.
- Data show that chemicals not picked up individually by one 3D-skin assay are picked by the other one which results in an overall **sensitivity of 92%**
- The HET-MN enables investigation of systemic availability of test compounds
- Based on these findings, these three novel genotoxicity assays have a great potential to complete the current *in vitro* battery (Ames, *in vitro*-MN)

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