

WHERE  
insights LEAD TO  
answers



## 3D SKIN-BASED GENOTOXICITY ASSAYS

Shambhu Roy, MSc, PhD, DABT, ERT

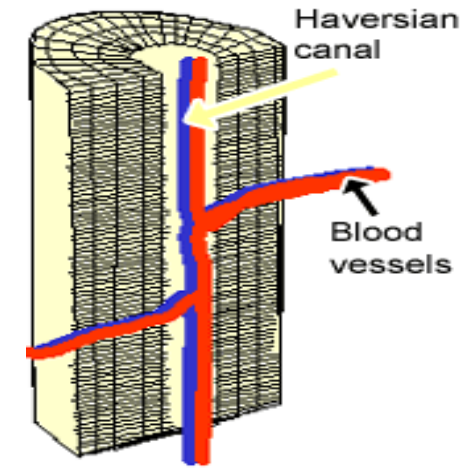
# Topics for Discussion

---

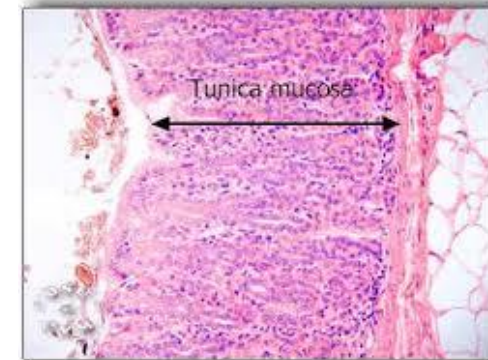
- What is 3D test system
- Why 3D test system needed
- 3D skin model
- Regulatory requirement for testing of cosmetic ingredients
- Qualification and validation of 3D skin-based genotoxicity assay
- Sample data
- Strategic fit of the 3D-based genotoxicity assay

# 3D Test System

- Structure with height, width, and depth or thickness
- Tissue structure in the organisms are 3 Dimensional



Bone



Intestinal wall

# Biological Relevance as a Test System

---

**2D cell cultures** limited in reflecting the in vivo situation:

For example,

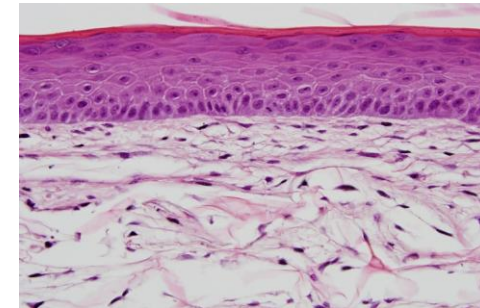
- Compared to native tissue, 2D cultures have less than 1% of cell density per volume and cell-to-cell contacts.
- Proliferation rate high



**2D tissue culture**

**3D tissue**

- Proliferation, morphology, function close to in vivo-like
- Suited as complex test systems for higher tier testing
- Route of exposure
- Intrinsic metabolic capacity

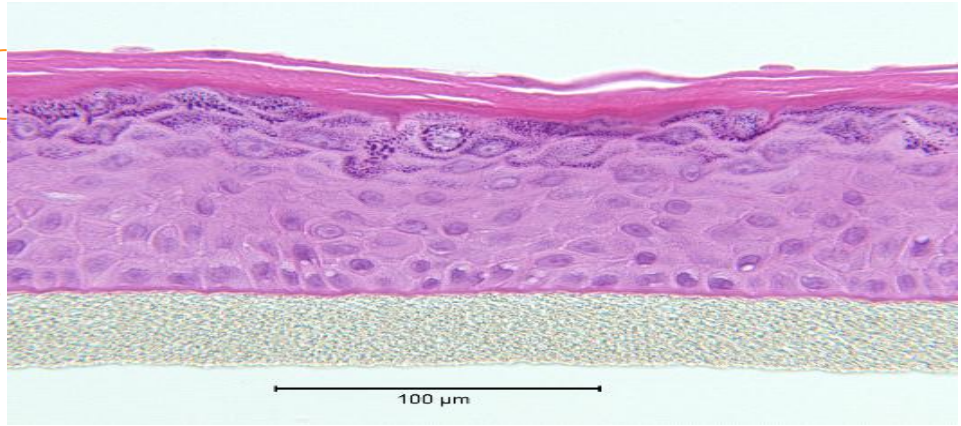


**3D tissue culture**

# 3D Skin Model

Stratum corneum

Stratum  
germinativum

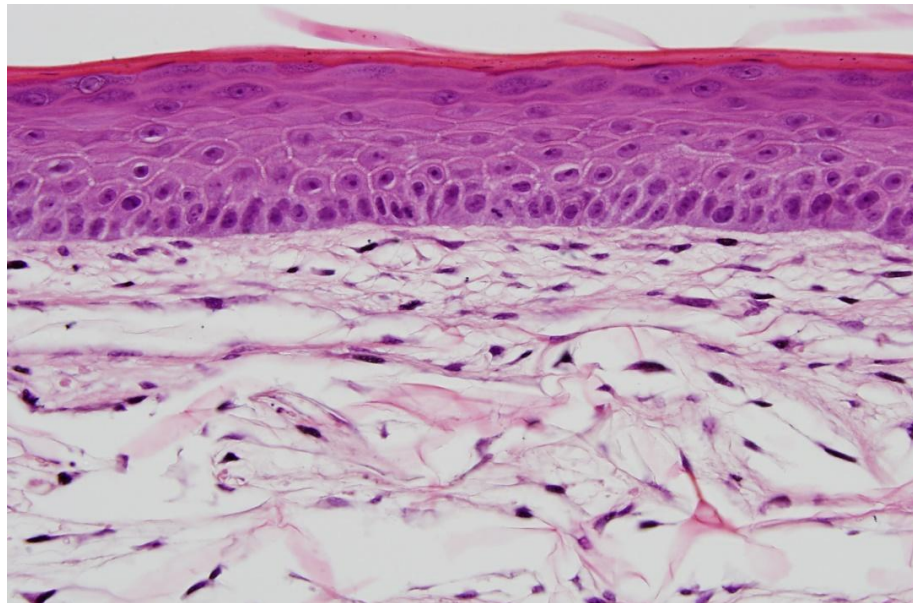


**Epidermis Model**

Source: MatTeK

Epidermis

Dermis



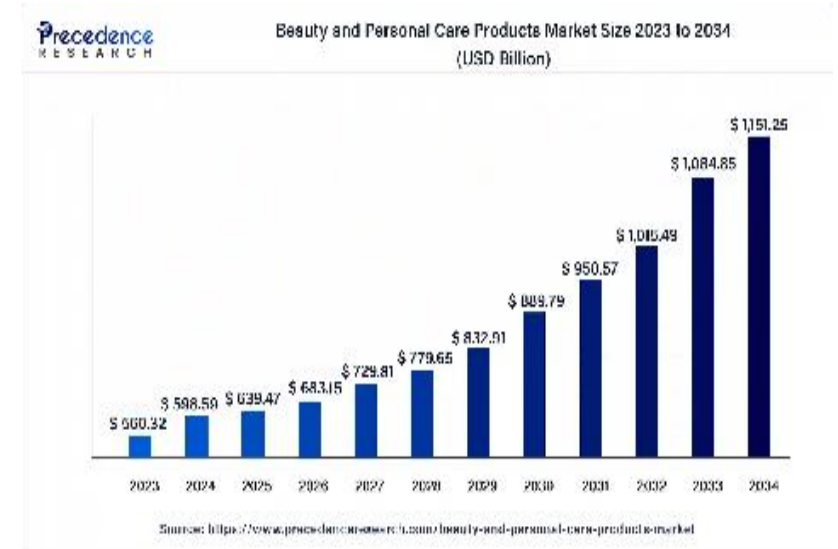
**Full thickness**

Source: Phinion, Henkel

# Regulatory requirement for Testing Cosmetics ingredients (EU 7th amendment)

- De-risk in vitro positive results
- 3Rs compliance
- European Union (EU) : 2009 Ban on the sale of animal tested cosmetics
- EU consumer markets: 2013 Imported cosmetics ingredients tested on animals were phased out
- Brazil and India: 2014 Ban on animal testing for cosmetics
- New Zealand: 2015 Ban on animal testing for cosmetics
- May 2018 the European Parliament voted for the EU and its Member States to work towards a UN convention against the use of animal testing for cosmetics

**Therefore, an alternative *in vitro* testing model that is equivalent to animal is needed for safety assessment of cosmetic ingredients.**



# Validation of 3D skin- based genotoxicity Assay

---

Our legacy facility 'BioReliance' participated in validation program initiated by Cosmetic Europe (CosEU), previously COLIPA, and developed:

- 3D skin micronucleus Assay (Reconstructed Skin Micronucleus Assay or RSMN Assay)
- 3D Skin Comet Assay (Reconstructed Skin Comet Assay or RSCOMET Assay)

At Inotiv we performed bridging studies to make sure that our new facility is well suited to perform this assay and generated the historical data. Our technical team largely remains the same to the legacy lab.

# 3D skin Genotoxicity Validation Program

---

- 52 chemicals were selected from the ECCVAM list of Reference test articles
- All chemicals were coded and distributed by third party randomly
- Test articles were direct and indirect genotoxicants and cytotoxicants
  - > Various modes of action known
- Study was conducted under same protocol as per Respective OECD testing guidelines

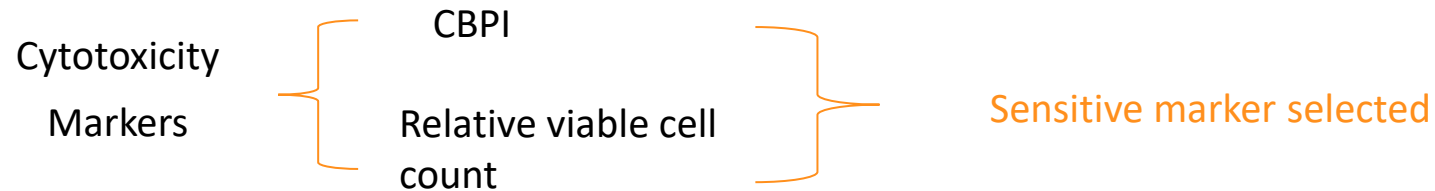
Original Manuscript Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays Stefan Pfuhler<sup>1,\*</sup>, Thomas R. Downs<sup>1</sup>, Nicola J. Hewitt<sup>2</sup>, Sebastian Hoffmann<sup>3</sup>, Greg C. Mun<sup>4</sup>, Gladys Ouedraogo<sup>5</sup>, Shambhu Roy<sup>6</sup>, Rodger D. Curren<sup>4</sup> and Marilyn J. Aardema<sup>7</sup> (*Mutagenesis*, 2021, 36, 1–17)

# Unique Testing Design

---

Testing design is based on OECD 487 (2023)

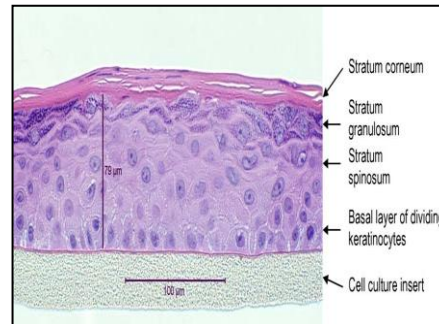
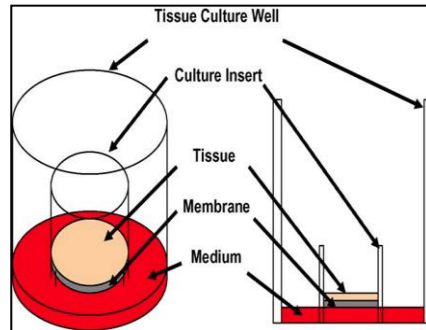
- Dosing: Topical, (similar to cosmetic use)
- Preferred solvent = Acetone, Ethanol
- Top dose = 100 mg/mL, unless limited by solubility
- 3 Tissues per dose
- Data evaluation similar to in vitro MN assay



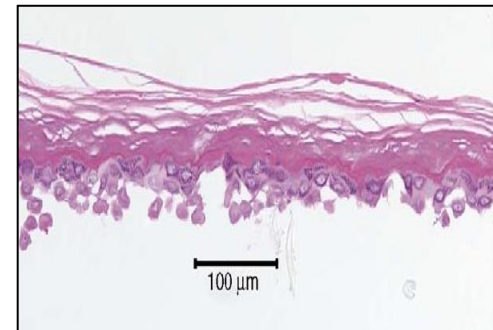
# Experimental Design (RSMN-Assay)



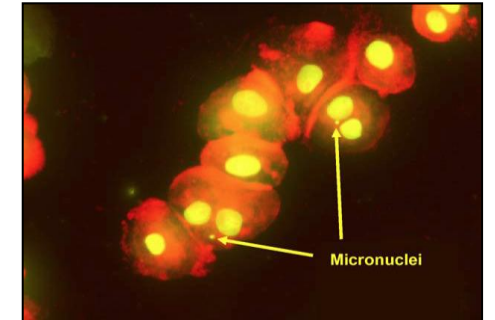
MatTek Corp



Pre trypsin



Post trypsin



AO stained cells

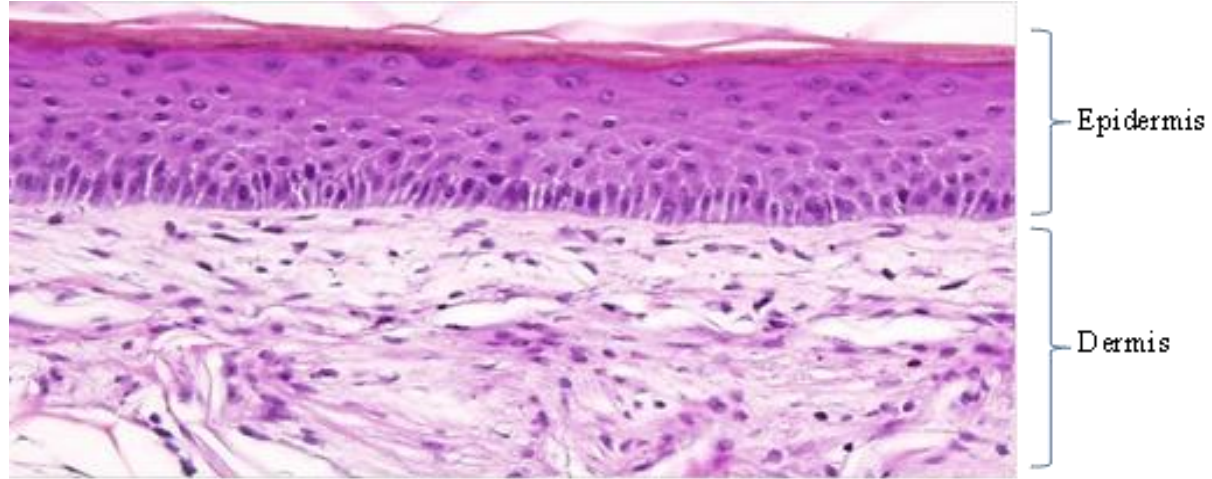
Histological section HE  
staining

Curren, *et al.* Mutation Res. 607, 2006; Dahl *et al.* Mutation Res. 720, 2011 (photocatalog)

# RSCOMET Assay (Reconstructed Skin Comet Assay)

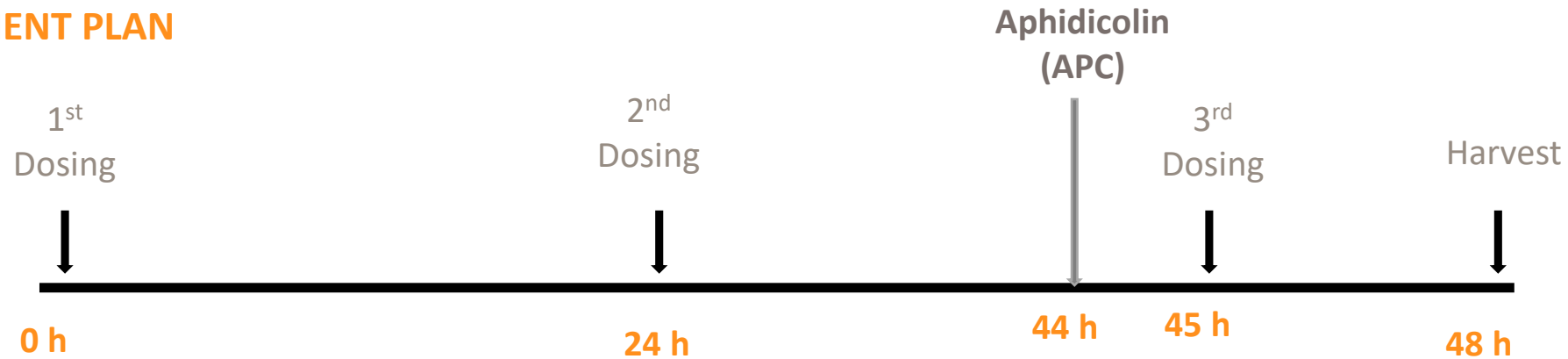


Phenion®  
Full-Thickness (FT) Skin Model



Histological section HE staining

## TREATMENT PLAN

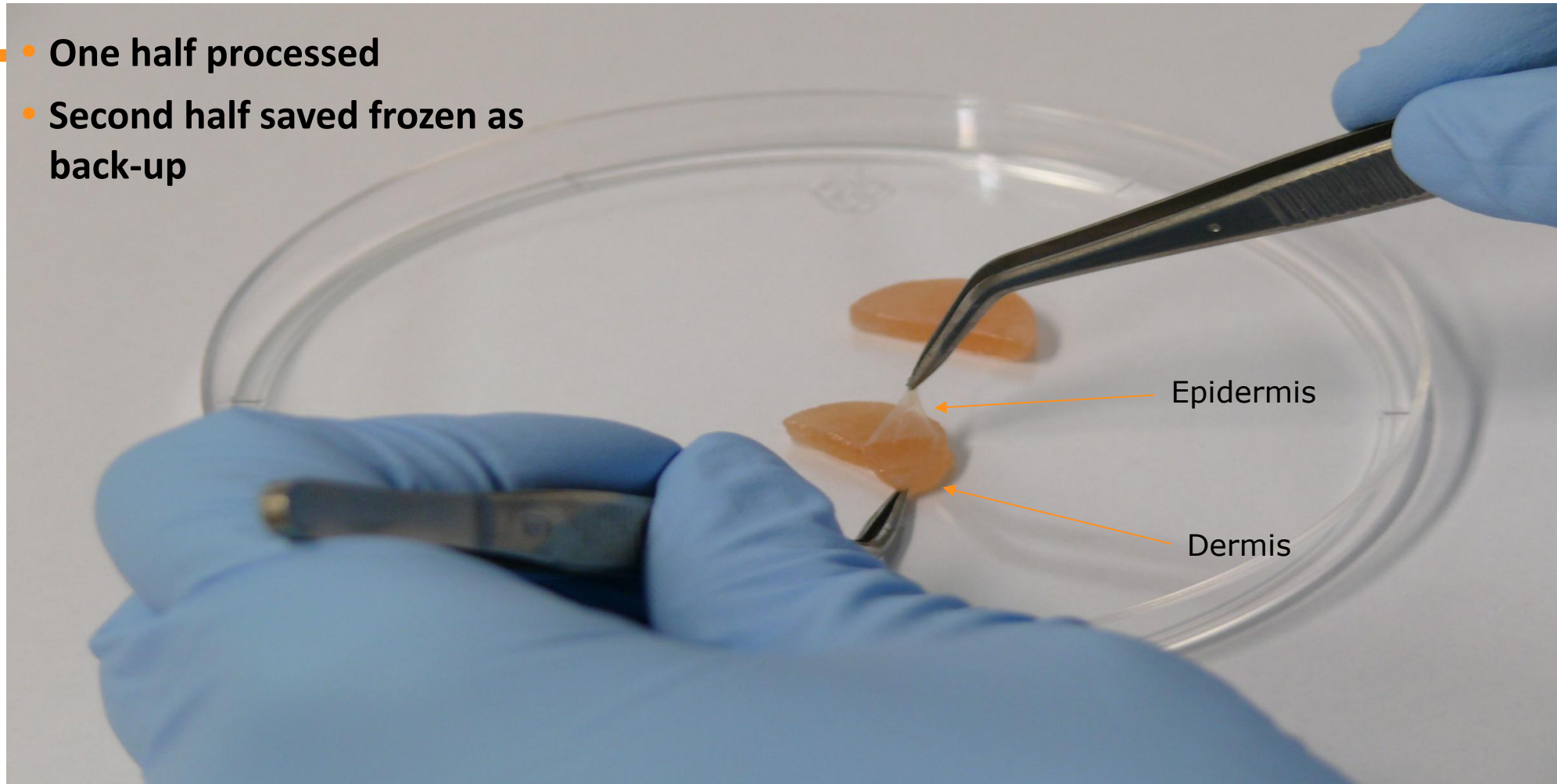


Definitive assay

Confirmatory Assay

# Separation of Epidermis and dermis

- One half processed
- Second half saved frozen as back-up



# CosEU Validation outcome –dermal route

| Parameter       | 3D Skin Comet Assay | RSMN |
|-----------------|---------------------|------|
| Chemical Tested | 28                  | 39   |
| Sensitivity     | 73                  | 80   |
| Specificity     | 87                  | 87   |
| Accuracy        | 80                  | 84   |

**Sensitivity** increases to **89%** when 3D Skin Comet assay and RSMN are combined in an endpoint specific battery approach.

*IWGT Nov 2017: Assays "sufficiently validated to move towards the development of individual OECD TG"*

# Comparison of Data in various studies in our Lab-Fragrance and Flavoring chemicals (2014 to 2018)

| Test Article | Ames     | Condition                     | in vitro MN | Condition                 | 3D Skin MN | in vivo MN |
|--------------|----------|-------------------------------|-------------|---------------------------|------------|------------|
| X1           | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X2           | Negative |                               | Positive    | HPBL; 4hr +/- S9          | Negative   | Negative   |
| X3           | Negative |                               | Positive    | HPBL: 3hr +/- S9, 24h -S9 | Negative   | Negative   |
| X4           | Negative |                               | Positive    | 3 hr arm +S9              | Negative   | Negative   |
| X5           | Positive | TA1537 -S9                    | Equivocal   |                           | Negative   | Negative   |
| X6           |          |                               | Positive    | HPBL: 4hr +/- S9, 24h -S9 | Negative   | Negative   |
| X7           | Negative |                               | Positive    | HPBL: 4hr +/- S9, 24h -S9 | Negative   | Negative   |
| X8           | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X9           | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X10          | Negative |                               | Positive    | HPBL; 24 hr - S9          | Negative   | Negative   |
| X11          | Positive | WP2 uvrA +/-S9;<br>TA1537 +S9 | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X12          | Positive | TA100 +S9                     | Positive    |                           | Negative   | Negative   |
| X13          | Negative |                               | Equivocal   | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X14          | Positive | TA1535 +S9                    | Negative    |                           | Negative   | Negative   |
| X15          | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X16          | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X17          | Negative |                               | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |
| X18          | Positive | TA 100 and WP2<br>uvrA -S9;   | Positive    | HPBL; 3hr +/- S9          | Negative   | Negative   |

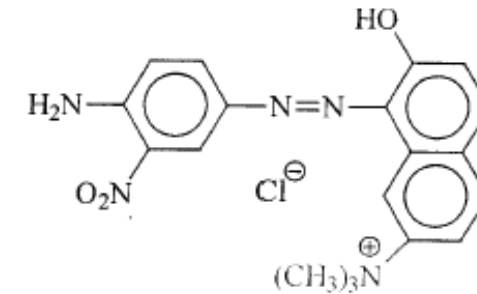
## Success story of Basic Brown 17 (B007) - a Hair Dye (Ref: SCCS/1531/14 Revision of 18 June 2014)

Exclusively used in the absence of H<sub>2</sub>O<sub>2</sub> as a direct hair dye

Marketed as Sienna Brown

CF = C<sub>19</sub>H<sub>20</sub>N<sub>5</sub>O<sub>3</sub> + Cl<sup>-</sup>

MW = 401.85



|                                          |                                                                                                                               |
|------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|
| Acute oral toxicity                      | LD50 ~16 mg/kg of BW                                                                                                          |
| Skin irritation                          | the test substance showed some irritant potential to the rabbit skin.                                                         |
| Eye irritation                           | test substance showed some irritant potential to the rabbit eye.                                                              |
| Local Lymph Node Assay (LLNA)            | Unacceptable vehicle, dose low, conclusion on sensitisation cannot be drawn                                                   |
| Dermal absorption in pig                 | Study performed very well, data is acceptable                                                                                 |
| Sub-chronic (90 days) toxicity (oral)    | NOAEL - 60 mg/kg bw/day                                                                                                       |
| Bacterial gene mutation assay (standard) | Positive - biologically relevant and dose dependent increase in the number of revertant colonies in strains TA98 and TA 1537. |
| Mouse Lymphoma assay (tk locus)          | Negative up to ~200 µg/mL although 10-20% survival was not achieved                                                           |
| Mouse Lymphoma assay (HPRT locus)        | Negative up to ~200 µg/mL although 10-20% survival was not achieved                                                           |
| In vitro micronucleus test (V79 cells)   | Negative up to 10 mM                                                                                                          |
| RS COMET (Phenion® Full-Thickness Skin)  | Negative 130 µg/cm <sup>2</sup> (8000 µg/ml): not clastogenic and mutagenic                                                   |

Most of the studies were performed around 2000, RS COMET in 2014

# SCCS CONCLUSION

---

On the basis of a weight of evidence approach, the positive result in the gene mutation test in bacteria is not confirmed in three different tests, measuring gene mutations in mammalian cells. Basic Brown 17 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Contact:

European Commission Health & Consumers Directorate C: Public Health Unit C2 –  
Health Information/ Secretariat of the Scientific Committee Office: HTC 03/073

L-2920 Luxembourg

[SANCO-C2-SCCS@ec.europa.eu](mailto:SANCO-C2-SCCS@ec.europa.eu)

# Regulatory Updates



| Validation                                                            |           |                                                                               |                                                                                                              |
|-----------------------------------------------------------------------|-----------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|
| Peer-review                                                           |           |                                                                               |                                                                                                              |
| Date                                                                  | Stage     | Comment                                                                       | Documents/Links                                                                                              |
| Jan 2025                                                              | Finalised | ESAC working group has been established                                       |                                                                                                              |
| Feb 2025                                                              | Finalised | ESAC Opinion on the Scientific Validity of the RS Comet and RSMN Test Methods | <a href="#">ESAC Opinion No. 2025-01 of 24 January 2025</a><br><a href="#">RS Comet Submission Documents</a> |
| Recommendation                                                        |           |                                                                               |                                                                                                              |
| Regulatory acceptance/Standards                                       |           |                                                                               |                                                                                                              |
| Tracking System for Alternative methods towards Regulatory acceptance |           |                                                                               |                                                                                                              |
| This site is managed by the Joint Research Centre                     |           |                                                                               |                                                                                                              |
| Contact us                                                            |           | About us                                                                      |                                                                                                              |
| <a href="#">Contact information of TSAR</a>                           |           | <a href="#">Information about TSAR</a>                                        |                                                                                                              |
| European Commission                                                   |           | Language policy                                                               |                                                                                                              |
| <a href="#">Contact the European Commission</a>                       |           | <a href="#">Follow the European Commission on social media</a>                |                                                                                                              |
|                                                                       |           | <a href="#">Cookies</a>                                                       |                                                                                                              |

<https://tsar.jrc.ec.europa.eu/test-method/tm2020-01>

## COM Guidance : Genotoxicity testing using 3D models Published 18 July 2024

<https://www.gov.uk/government/publications/genotoxicity-testing-using-3d-models/genotoxicity-testing-using-3D-models>

# Strategic fit of RSMN and RSCOMET assays

These assays can be used in combination or alone depending on the scenario, since these 3D models are suitable for 'endpoint-specific' follow-up of positives from standard *in vitro* testing battery. For example:

| Assay Types                     | Mutation | Structural chromosome damage | Numerical chromosome damage | Follow-up Studies |
|---------------------------------|----------|------------------------------|-----------------------------|-------------------|
| Standard <i>in vitro</i> Assays | Positive | Positive                     | Negative                    | 3D skin Comet     |
|                                 | Negative | Positive                     | Positive                    | 3D skin MN        |

- *in vitro* mutagenicity positives - Recommended to do 3D comet
- *in vitro* clastogenicity and/ or aneugenicity positives - Recommended to do 3D MN
- *in vitro* mutagenicity & clastogenicity and/or aneugenicity positives - Recommended to perform both 3D comet and 3D MN

Explore our Genetic Toxicology Services

