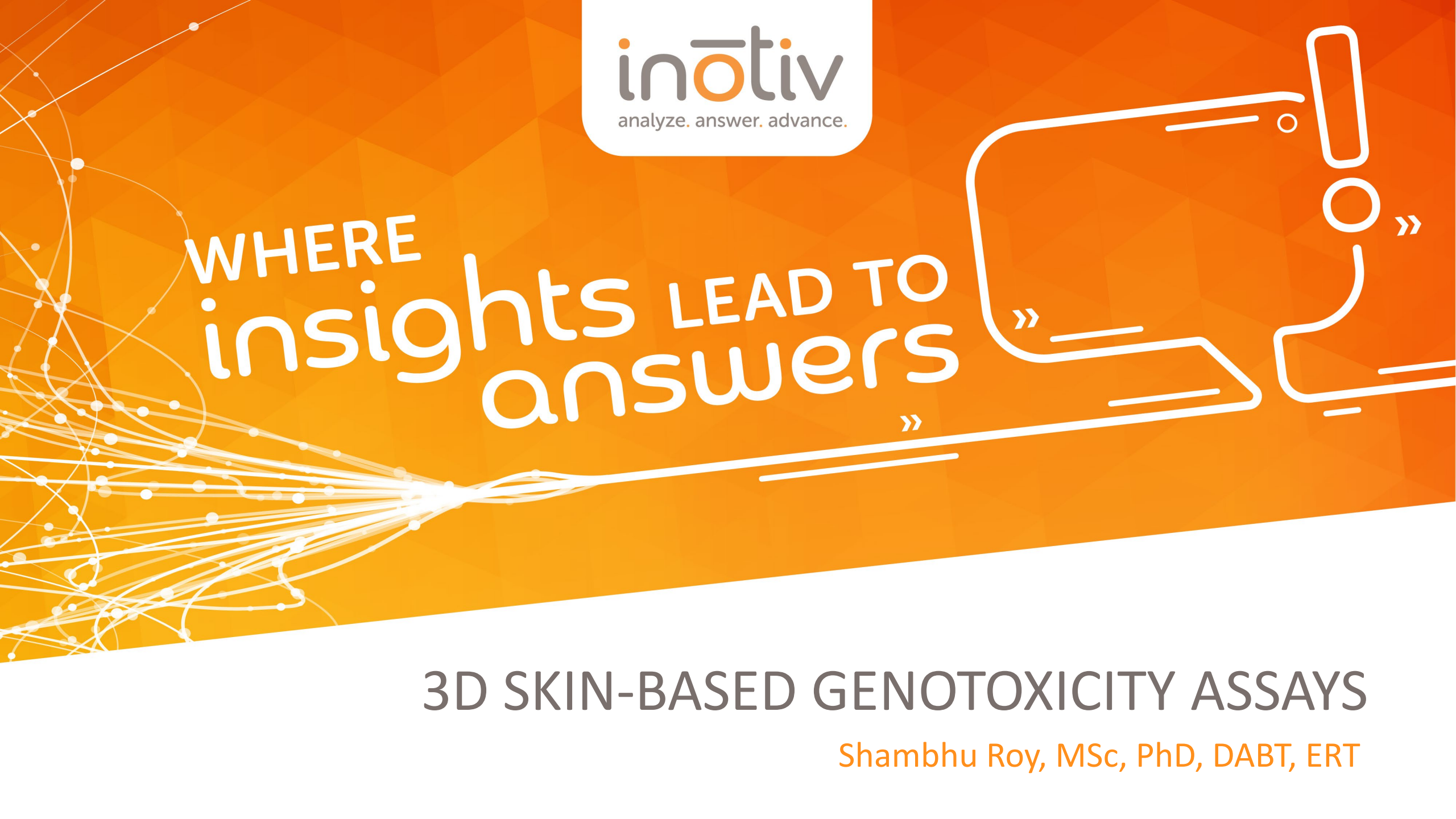




WHERE
insights LEAD TO
answers



3D SKIN-BASED GENOTOXICITY ASSAYS

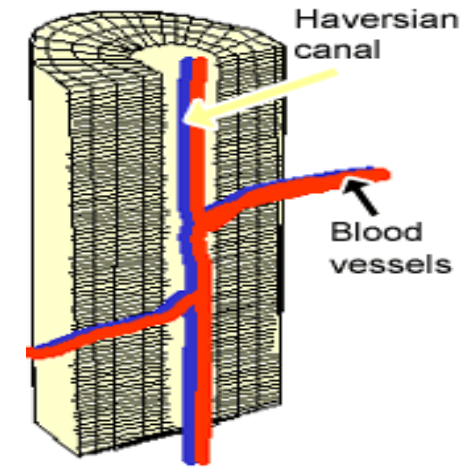
Shambhu Roy, MSc, PhD, DABT, ERT

Topics for Discussion

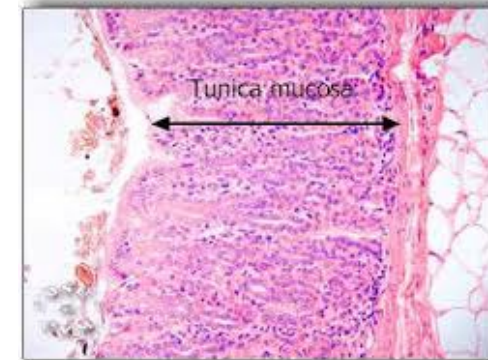
- What is 3D test system
- Why 3D test system needed
- 3D skin model
- Regulatory requirement for testing of cosmetic ingredients
- Qualification and validation of 3D skin-based genotoxicity assay
- Sample data
- Strategic fit of the 3D-based genotoxicity assay

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,

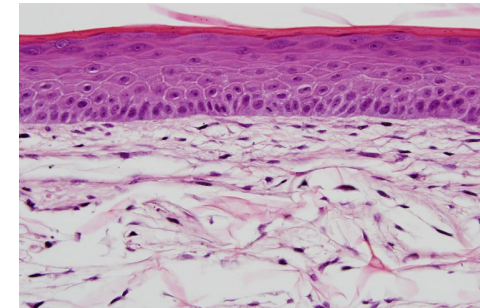
- Compared to native tissue, 2D cultures have less than 1% of cell density per volume and cell-to-cell contacts.
- Proliferation rate high

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



2D tissue culture

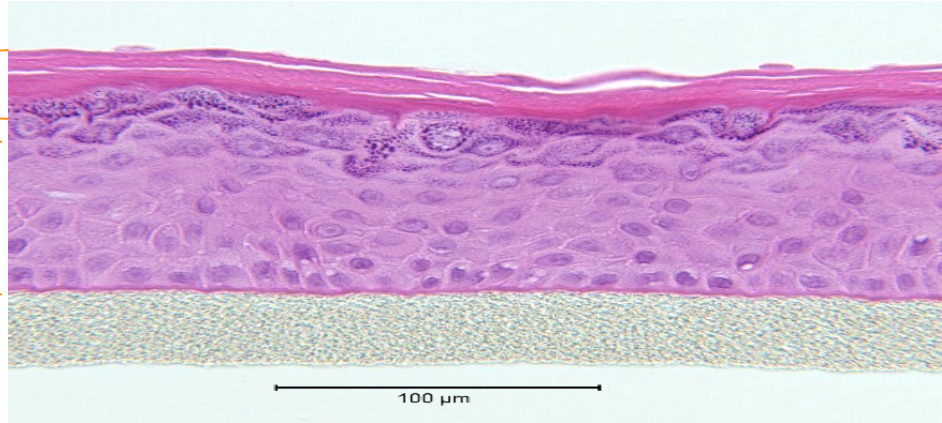


3D tissue culture

3D Skin Model

Stratum corneum

Stratum
germinativum

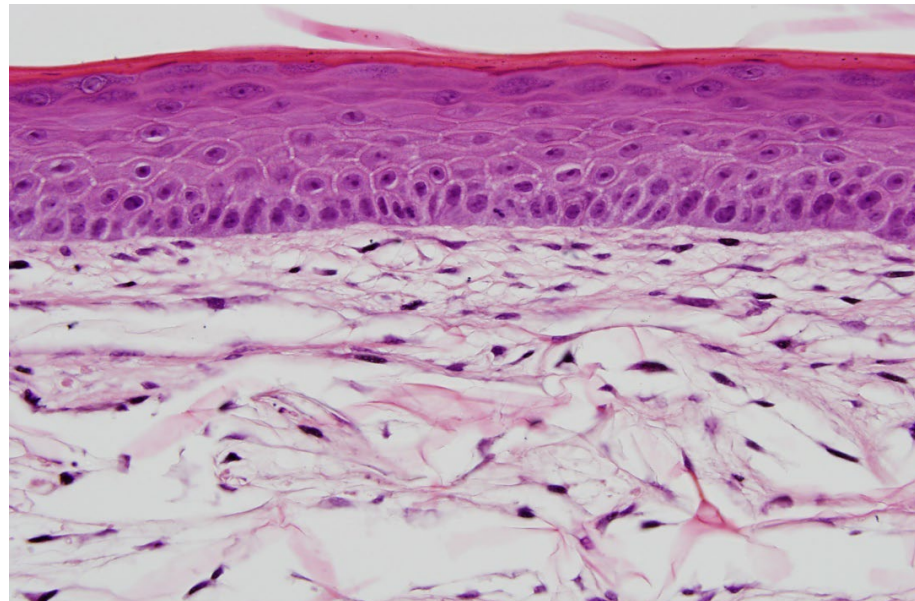


Epidermis Model

Source: MatTeK

Epidermis

Dermis

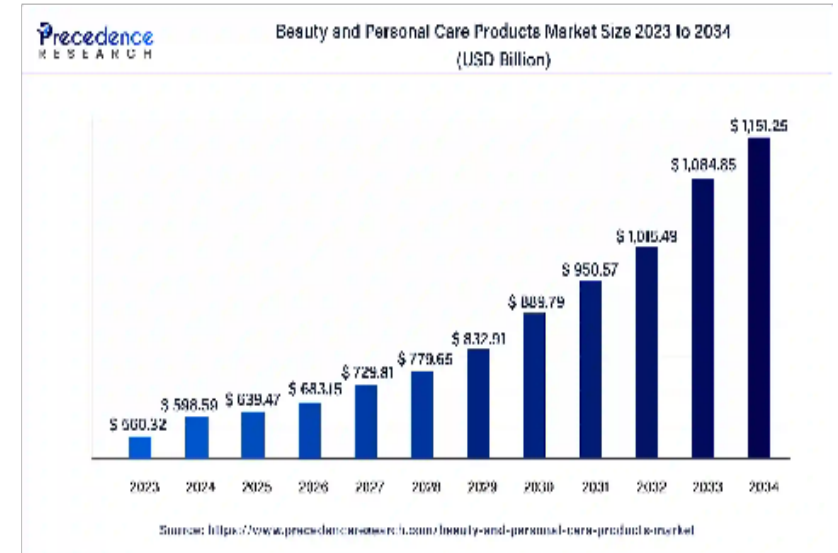


Full thickness

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^{1,*}, Thomas R. Downs¹, Nicola J. Hewitt², Sebastian Hoffmann³, Greg C. Mun⁴, Gladys Ouedraogo⁵, Shambhu Roy⁶, Rodger D. Curren⁴ and Marilyn J. Aardema⁷ (*Mutagenesis*, 2021, 36, 1–17)

Unique Testing Design

Testing design is based on OECD 487 (2023)

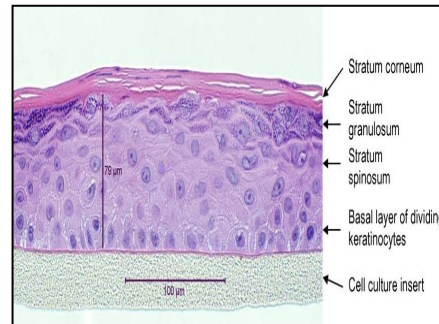
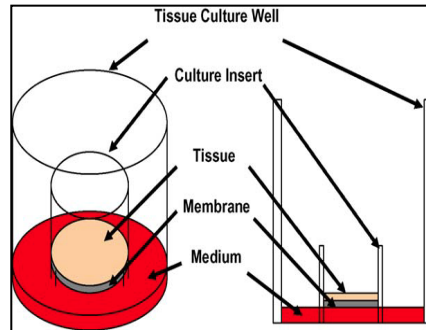
- Dosing: Topical, (similar to cosmetic use)
- Preferred solvent = Acetone, Ethanol
- Top dose = 100 mg/mL, unless limited by solubility
- 3 Tissues per dose
- Data evaluation similar to in vitro MN assay



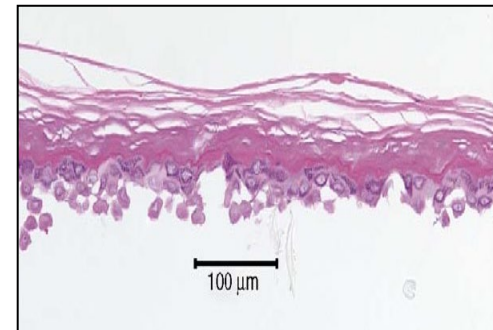
Experimental Design (RSMN-Assay)



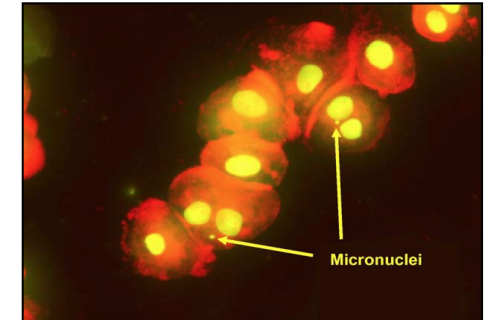
MatTek Corp



Pre trypsin



Post trypsin



AO stained cells

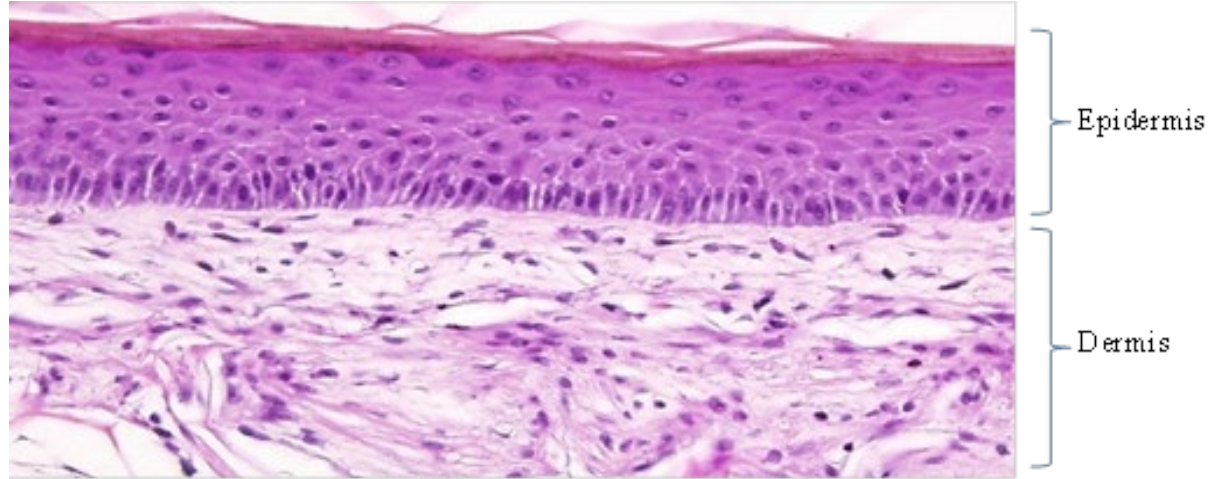
Histological section HE
staining

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

RSCOMET Assay (Reconstructed Skin Comet Assay)

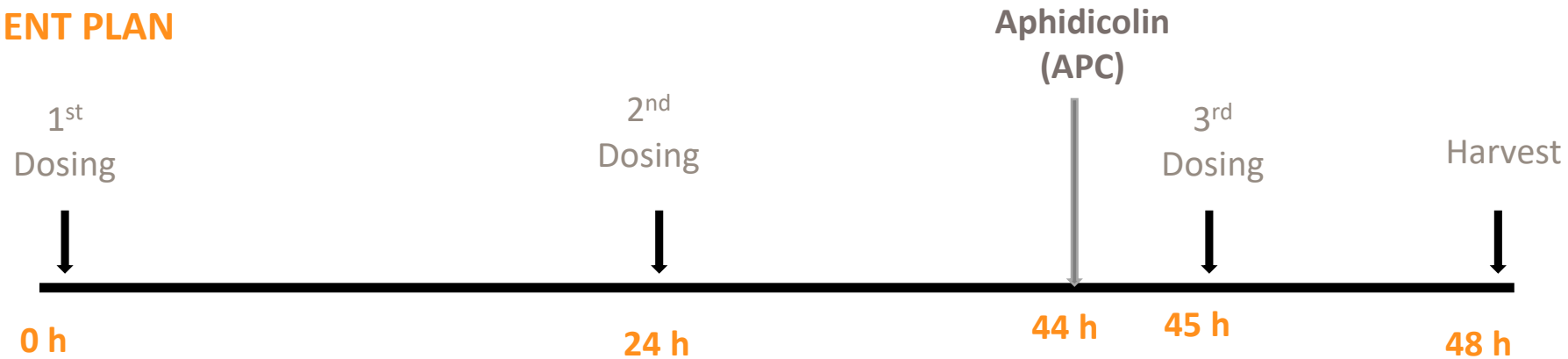


Phenion®
Full-Thickness (FT) Skin Model



Histological section HE staining

TREATMENT PLAN

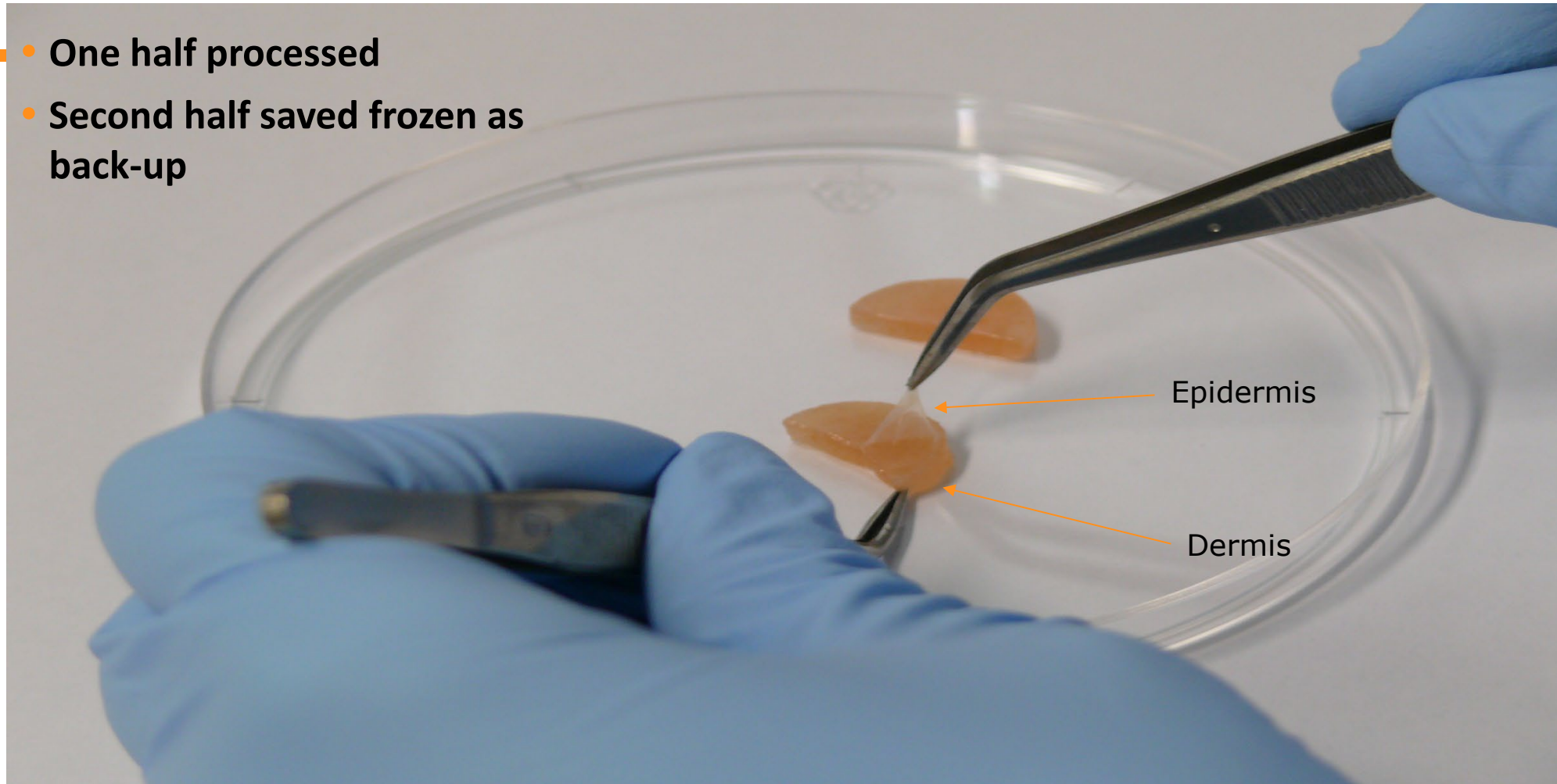


Definitive assay

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; 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 uvrA -S9;	Positive	HPBL; 3hr +/- S9	Negative	Negative

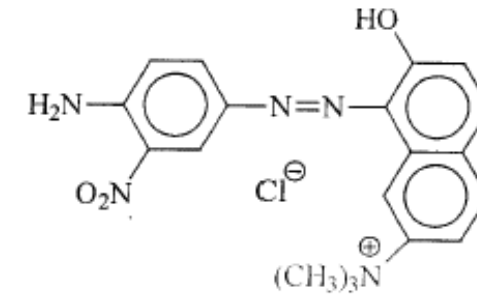
Success story of Basic Brown 17 (B007) - a Hair Dye (Ref: SCCS/1531/14 Revision of 18 June 2014)

Exclusively used in the absence of H₂O₂ as a direct hair dye

Marketed as Sienna Brown

CF = C₁₉H₂₀N₅O₃ + Cl⁻

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 ² (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

Regulatory Updates

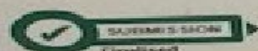
3D Reconstructed Human Skin Comet assay

TM Status: [Open](#)
Test Method Number: TM2020-01 (EU)
Short Name of TM: RS Comet assay
Responsible Organisation: EURLECVAM - European Union [\[E\]](#)

3D Reconstructed Human Skin Micronucleus assay (RSMN)

TM Status: [Open](#)
Test Method Number: TM2020-04 (EU)
Short Name of TM: RSMN
Responsible Organisation: EURLECVAM - European Union [\[E\]](#)

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	ESAC Opinion No. 2025-01 of 24 January 2025 RS Comet Submission Documents

Recommendation

Regulatory acceptance/Standards

Tracking System for Alternative methods towards Regulatory acceptance

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<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

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