Advances in Genotoxicity - Reconstructed Skin Comet

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ASCCT Webinar

21/01/2022

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Agenda

1. Background
2. Concept of the use of 3D skin tissue equivalents in genotoxicity testing
3. Reconstructed skin comet assay
    1. Principle
    2. Validation exercise
    3. Regulatory acceptance
4. Reconstructed skin micronucleus assay
5. Strategic fit of assays in testing strategies
6. Summary/outlook

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Introduction

Regulatory landscape

- Industry sectors in the European Union (EU) which restrict or ban animal experiments:
  - REACh 1907/2006, EU Cosmetics Directive 1223/2009/EC
- A growing number of regions ban animal testing for the safety assessment of cosmetics e.g.
  - Ban: EU, Australia, NZ, UK, Columbia
  - Phasing out: Japan, US, Canada
  - China started to allow *in vitro* only approaches for specific scenarios/cosmetic product categories

Assessment challenge

- Classical *in vitro* genotoxicity test battery is known to be very sensitive for *in vivo* genotoxicants/carcinogens
- However, the 'test battery' approach of combining assays leads to a reduced specificity – increase in fraction of 'misleading positives'

Introduction

- Classical *in vitro* genotoxicity tests are based on 2D cell cultures

- 2D cell cultures limited in reflecting the *in vivo* situation e.g.,
  - *"2D cultures have less than 1% of both cell density per volume and cell-to-cell contacts when compared to native tissues"*
  - Highly proliferative

- 3D tissue
  - Key parameters similar to the *'in vivo'* situation: proliferation, morphology, function
  - Suited as complex test systems for higher tier testing
    - Route of exposure
    - Intrinsic metabolic capacity

Approach

Cosmetics Europe´s animal-free strategy for genotoxicity testing

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Cosmetics Europe Genotoxicity program

1. Improve predictivity of current assays – in specific MNvit
2. Develop and validate new *in vitro* assays
3. Investigate xenobiotic metabolism of respective test systems

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Cosmetics Europe Genotoxicity program

1. Improve predictivity of current assays – in specific MNvit
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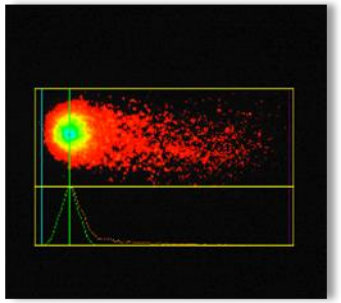
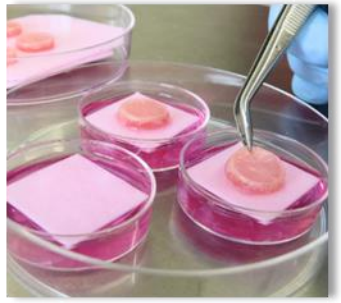
Dermal route

Dermal Route

3D Skin Comet

Reconstructed Skin Comet assay



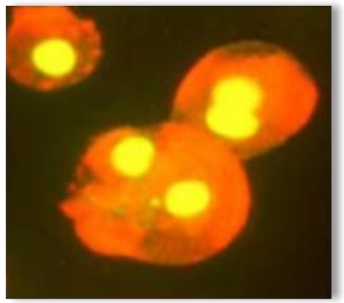


Phenion® Full-Thickness Skin Model

www.phenion.com

RSMN

Reconstructed Skin MicroNucleus test



EpiDerm™ (MatTek)

- First *in vitro* options for genotoxicity assessment of the dermal exposure route.
- A route relevant for cosmetics, household prod. etc.
- Skin tissues based on primary p53 competent cells of human origin.
- Gene and protein expression patterns similar to human native skin e.g. xenobiotic metabolism - utilization of external metabolizing systems/rat liver S9 mix not required.
- Topical application reflects barrier function of the skin and allows application of concentrations that may be toxic to 2D cultures.

Dermal route

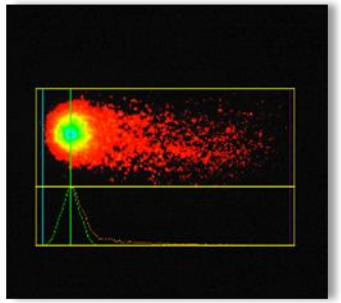
Dermal Route

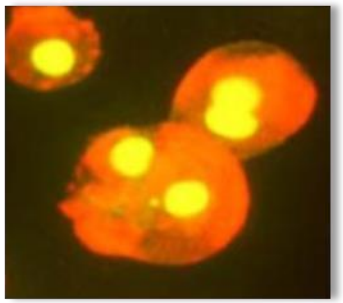
3D Skin Comet

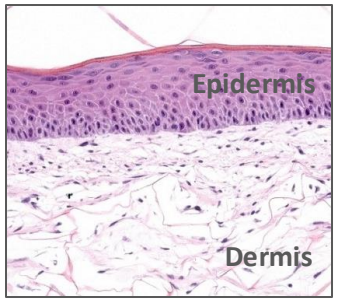
Reconstructed Skin Comet assay

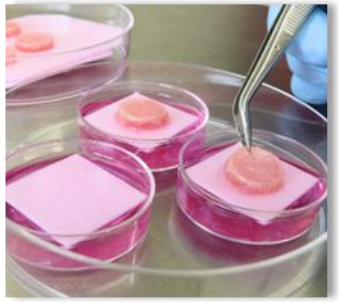
RSMN

Reconstructed Skin MicroNucleus test









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EpiDerm™ (MatTek)

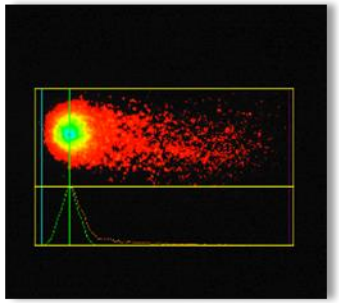
- Tissues have been selected from available ones based on assay specific performance criteria

Dermal route

Dermal Route

3D Skin Comet

Reconstructed Skin Comet assay



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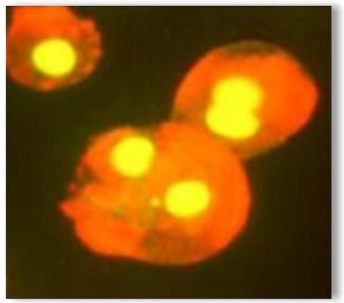
Reconstructed Skin MicroNucleus test



EpiDerm™ (MatTek)

- Test systems combined with classical read-outs.
- Battery of two assays addresses all three endpoints.

Assay	Mutation	Structural	Numerical
		Chromosome damage	
RS Comet	x	x	
RSMN		x	x

- Assays are intended to follow up on initial positive findings.

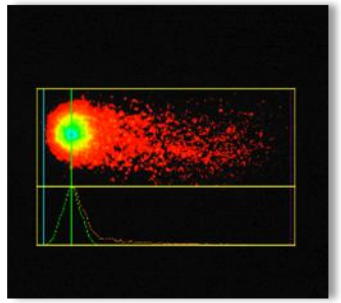
External reviews of 3D genotoxicity skin program

- IWGT WG “In vitro genotoxicity test approaches with better predictivity”, Basel, Switzerland 2009
- HESI Workshop “New Technologies”, Washington DC, USA 2012
- Expert Workshop on validation status and next steps for 3D skin based genotoxicity assays, Brussels, Belgium 2016
- CE Workshop on validation outcome of 3D skin-based genotoxicity assays and HET-MN, Brussels, Belgium 2017
- IWGT WG “Use of 3D Tissues in Genotoxicity Testing” Tokyo, Japan 2017

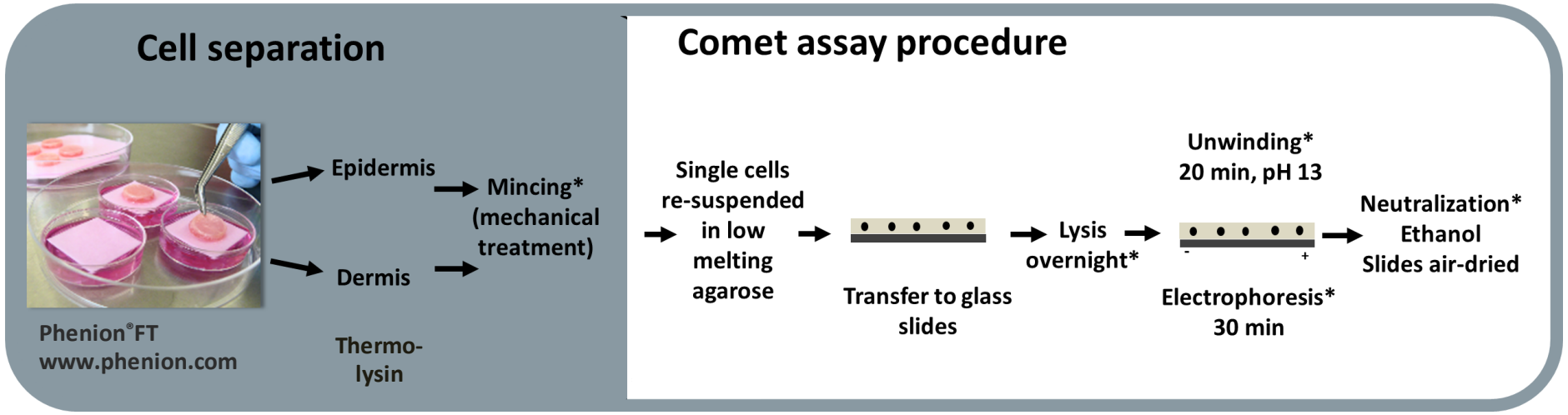
- ✓ 3D tissue models offer a more ‘*in-vivo*-like’ behavior for key parameters like cell viability, proliferation differentiation, morphology, gene and protein expression, and function. Therefore they provide a valuable complement to the classical ‘2D’ cell culture based assays.
- ✓ 3D tissue-based genotoxicity assays can be used as ‘2nd tier’ assays to follow up on positive results from standard *in vitro* assays.

Comet assay

- Does not rely on proliferating cells and allows for the investigation of DNA damage in any cell culture or tissue that can be subjected to single cell isolation.
- Widely use method for the detection of DNA damage in genotoxicity testing and human biomonitoring studies or environmental monitoring.
- An OECD test guideline developed in 2014 of the *in vivo* mammalian, alkaline comet assay no. 489 in 2014 (updated).
- The alkaline comet assays detects a wide range of DNA damage including modification that lead to gene mutation.
- Recent publications show the high sensitivity of *in vivo* alkaline comet assay for carcinogens that show gene mutation activity (comparably efficient as TGR).

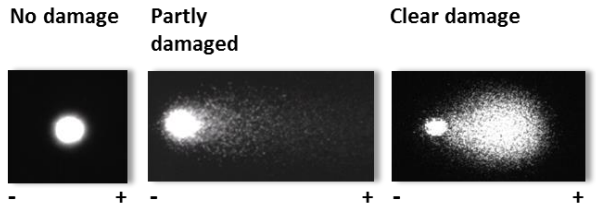


Test Principle – alkaline comet assay



Slide analysis

- Coding and staining (SYBR gold) of slides
- Semi-automated analysis (Comet Assay IV, Perceptive Instruments)
- Fluorescence intensity in comet tail (%tail DNA)



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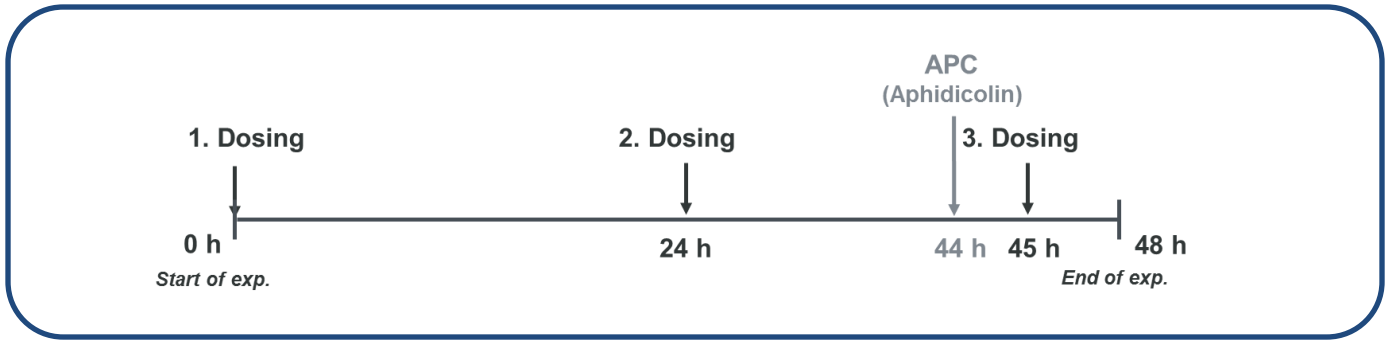
Experimental design



- Solvent control: acetone, 70% ethanol [v/v]
- Positive control: standard protocol - MMS, APC protocol - BaP
- At least three concentrations of test compound
- Three tissues per control/dose group

- Negative findings are further investigated in an APC experiment

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Cytotoxicity

- Protocol complemented with assays measuring general cellular toxicity as DNA damage also be triggered as a mechanism secondary to cytotoxicity
- Only a subset of dermal cells proliferate in both human native skin and full thickness skin tissues

→ Proliferation as measurement for general cytotoxicity not appropriate

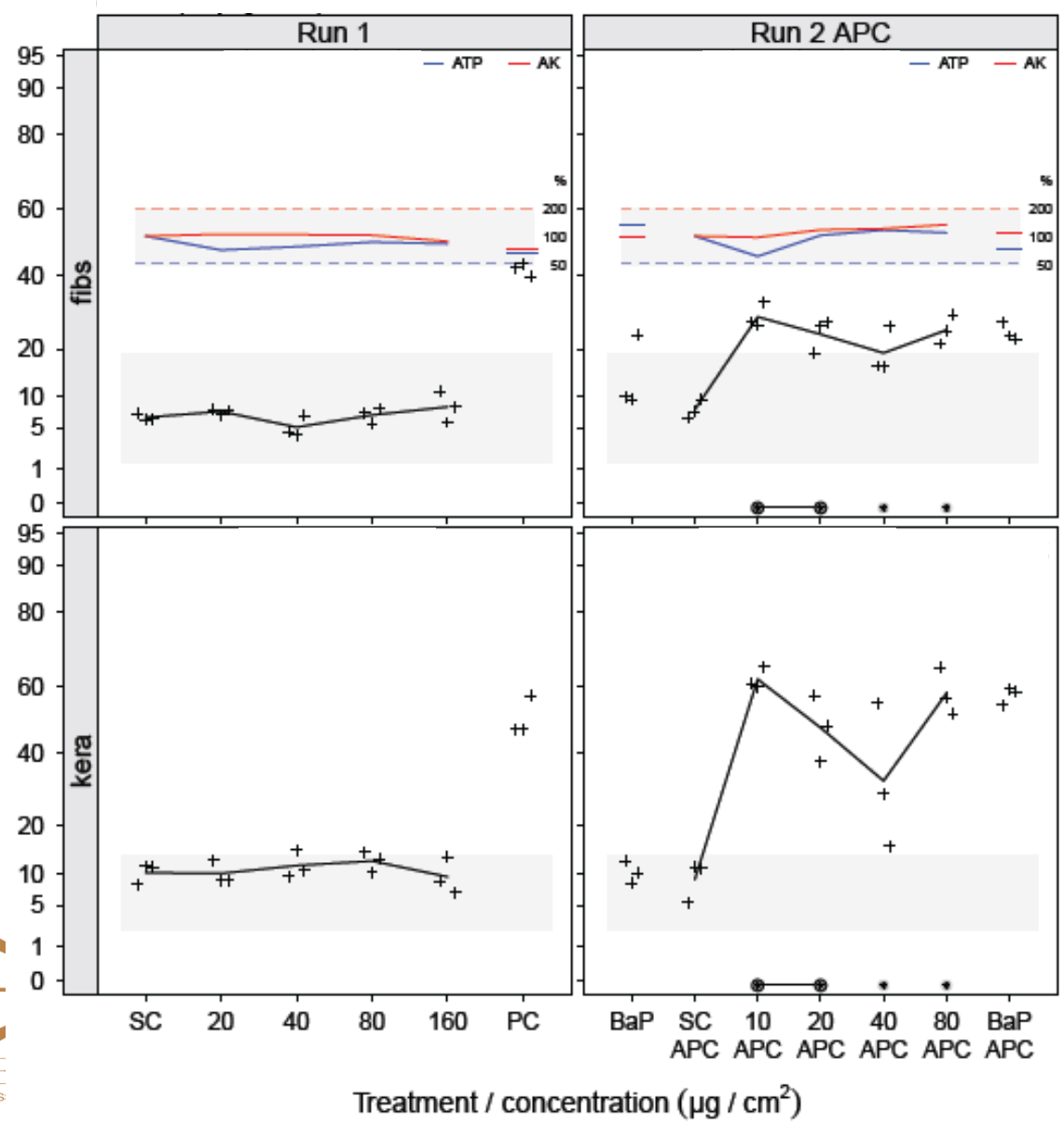
→ Proliferation not required to e.g. manifest read-out or monitor cell cyles

Release of adenylate kinase (AK) into the culture medium – tissue viability
Intracellular ATP concentration – energy status

Advantages

- Sensitive parameters
- Take effects into account that accumulate during 48 h exposure (AK) or that may arose shortly (ATP).
- Are detected in the same tissue used for genotoxicity measurement.

Study design



(1) Solubility study

(2) Dose range finder

- Maximum dose 1600 µg/cm^2

(3) At least 2 main experiments

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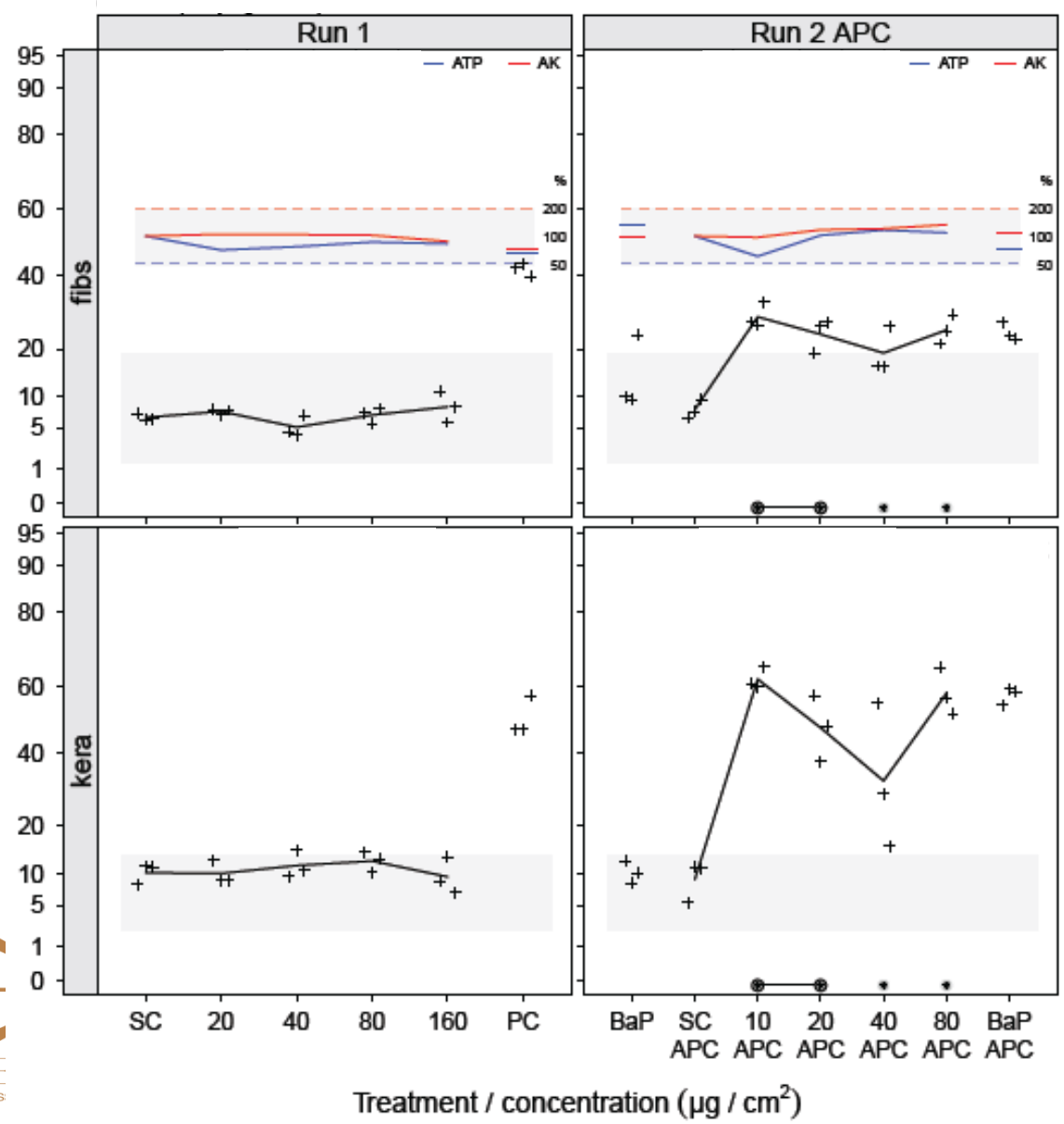
Read-outs



Cytotoxicity

Genotoxicity

HC= mean hSC + 2σ

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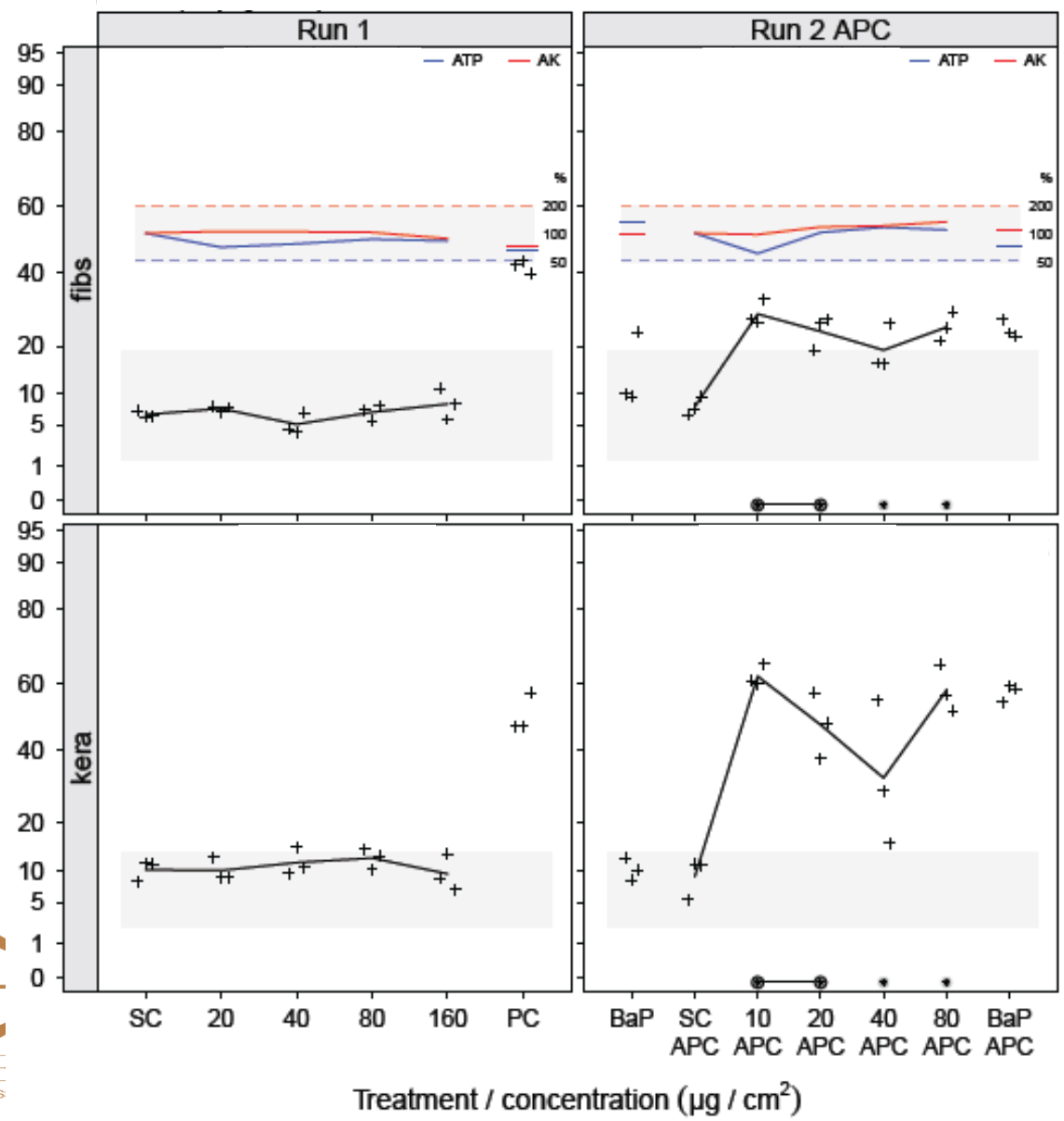
Validity criteria



- 3 valid dose groups based on cytotox.
  - ≥ 50% intracellular ATP content compared to SC
  - ≤ 200% adenylate kinase activity compared to SC
- SC ≤ 20% tail DNA
- PC 2x SC, MMS ≥ 15% to SC
  BaP ≥ 5% to SC

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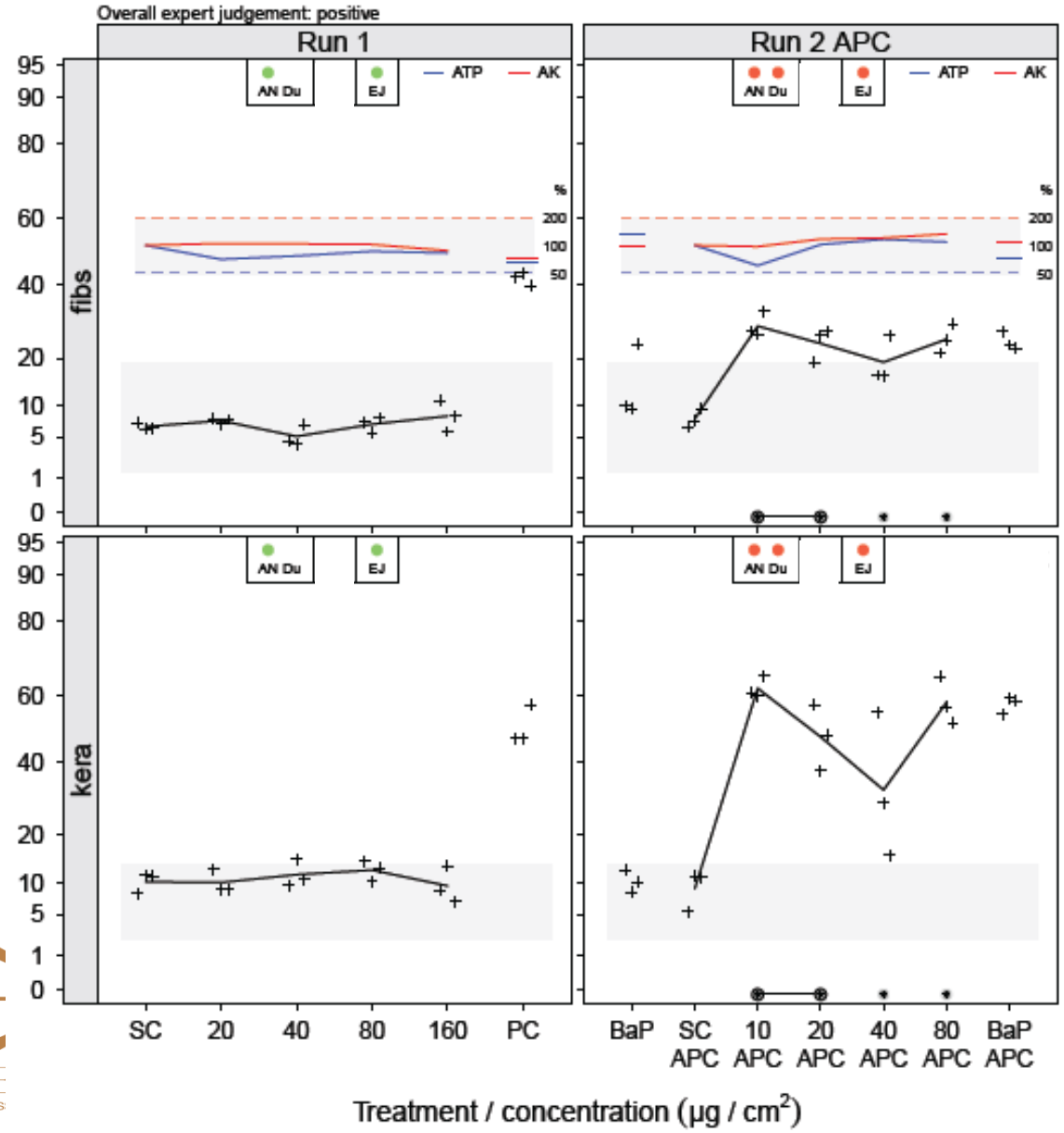
Data evaluation – Example Benzo(*a*)pyrene



Prediction model

- Transformation, ANOVA, Dunnett

Biological relevance

- Dose-dependency
- Reproducibility of effects
- Outside historical control range

Labs provided overall expert judgement

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Layout of validation

- **Phase 1**
  - Optimization and transferability
  - Limited number of known compounds
- **Phase 2**
  - Intra- and inter-laboratory reproducibility
  - Up to 18 double blinded compounds
- **Phase 3**
  - Extension of chemical space
  - Up to 30 additional coded chemicals

Validation exercise

- Independent selection of chemicals
  - True positive chemicals
  - True negatives
  - Misleading positives
- Independent statistical analysis

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Results - Expected negatives

Chemical	Lab A	Lab B	Lab C	Lab D	Lab E
Amitrole	neg				
Ampicillin sodium salt		neg			
N-butyl chloride	neg				
Curcumin		neg			
Cyclohexanone		neg		neg	neg
2,6-DAT		neg			
N,N-dicyclohexyl thiourea		neg			
Ethionamide	neg				
Di-(2-ethylhexyl) phthalate	neg		neg	neg	
Eugenol	neg		neg		neg
Glyoxal		neg			
D-Mannitol			neg		
Propyl gallate	neg	neg		neg	
Resorcinol		equiv			
Sodium xylene sulfonate			neg		
Tert-butylhydroquinone	neg				

Negative (neg)

- None of the criteria for a positive call apply

Positive (pos)

- All criteria for a positive call fulfilled

Equivocal (equiv)

- Some but not all criteria for a positive call fulfilled

Results - Expected positives

Chemical	Lab A	Lab B	Lab C	Lab D	Lab E
2-AAF		pos			
IQ	neg				
Azidothymidine		pos			
BaP	pos				
4-Chloroaniline		neg			
Cadmium chloride	pos	pos		neg	
Cyclopenta[c,d]pyrene	pos				
Cyclophosphamide		pos			
2,4-DAT		neg			
DMBA	pos	pos	pos		
EMS		pos			
ENU		pos	pos		pos
Etoposide			pos		
MNNG		pos			
Mitomycin C			pos	pos	pos

Negative (neg)

- None of the criteria for a positive call apply

Positive (pos)

- All criteria for a positive call fulfilled

Equivocal (equiv)

- Some but not all criteria for a positive call fulfilled

Reproducibility

Within laboratory - reproducibility

Chemical	Disconcordant	Concordant	Total	%
Lab A	0	8	8	100
Lab B	2	13	15	87
Lab C	0	8	8	100
Lab D	1	4	8	80
Lab E	0	4		100
All Labs	3	37	5	**93**

Between laboratory - reproducibility

Disconcordant	Concordant	Total	%
1	7	8	**88**

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Pfuhler et al., 2021

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Predictivity

Parameter	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Overall	Parameter
Chemicals considered	12	17	9	5	4	32	Chemicals considered
Sensitivity	80	80	100	50	100	**80**	Sensitivity
Specificity	100	94	100	100	100	**97**	Specificity
Accuracy	92	86	100	80	100	**92**	Accuracy

Pfuhler et al., 2021, based on final evaluation criteria

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Intended regulatory use - Case study

Basic Brown 17

- Hair dye - exclusively used in the absence of H_2O_2 as a direct hair dye

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Case study: Basic Brown 17
Information on genotoxicity

Available data	Follow up testing
• Ames - positive (+/- S9 mix) • MNvit V79 - negative	• MLA L5178Y - negative • HPRT L5178Y - negative
• No *in vivo* data available on genotoxicity or carcinogenicity	• Lack of genotoxic effects in terms of mutagenicity and clastogenicity **confirmed by 3D Skin Comet assay**

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Case study: Basic Brown 17

- Data submitted to the EU Scientific Committee on Consumer Safety (SCCS).
- Negative data of 3D Skin Comet assay were accepted in a weight-of-evidence approach.
- Basic Brown 17 was declared as 'Safe of use' in terms of genotoxicity (SCCS/1531/14, 24.03.2014).
- Two additional case studies on hair dye ingredients were successfully processed the same way (B34, A138).
- Since 2014 SCCS recommends using both the 3D Skin Comet assay and the RSMN to follow-up positive results from the *in vitro* standard test battery to build WoE in human relevant models.

Broader regulatory acceptance – Way to OECD TG

- IWGT WG "In vitro genotoxicity test approaches with better predictivity", Basel, Switzerland 2009
- HESI Workshop "New Technologies", Washington DC, USA 2012
- Expert Workshop on validation status and next steps for 3D skin-based genotoxicity assays, Brussels, Belgium 2016
- CE Workshop on validation outcome of 3D skin-based genotoxicity assays and HET-MN, Brussels, Belgium 2017
- IWGT WG "Use of 3D Tissues in Genotoxicity Testing" Tokyo, Japan 2017

- **OECD WNT – review of SPSF for guideline development. Paris, France 2019**
- **ECVAM peer-review (accepted into program)**
    - **Full submission of RS Comet and RSMN data in 2021**

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Studies to be contacted at Charles River Laboratories

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Acknowledgements

Cosmetics Europe TF Genotoxicity

Rolf Fautz, Arianna Giusti, Nicola Hewitt, Sebastian Hoffmann, Julia Kenny, Gladys Ouedraogo, Kerstin Reisinger, Brian Wall

Collaborators

P&G, USA: Tom Downs, Brenda Barnett

TNO, The Netherlands: Cyrille Krul, Astrid Reuss

L'Oreal, France: Gladys Ouedraogo

Henkel, Germany: Kerstin Reisinger

BfR/FU Berlin, Germany: Frank Henkler, Joop Brinkmann; Andre Said, Monika Schaefer-Korting

BASF, Germany: Markus Schulz, Veronica Blaz

Imperial College London, UK: Rob Edwards

University Duesseldorf: Ellen Fritsche

External Experts

David Kirkland
Raffaella Corvi
Rodger Curren
Jan van Benthem
Sebastian Hoffman
Ralph Pirow
Manfred Liebsch
Tom Slaga
Johannes Döhmer
Peter Kasper
Günter Speit

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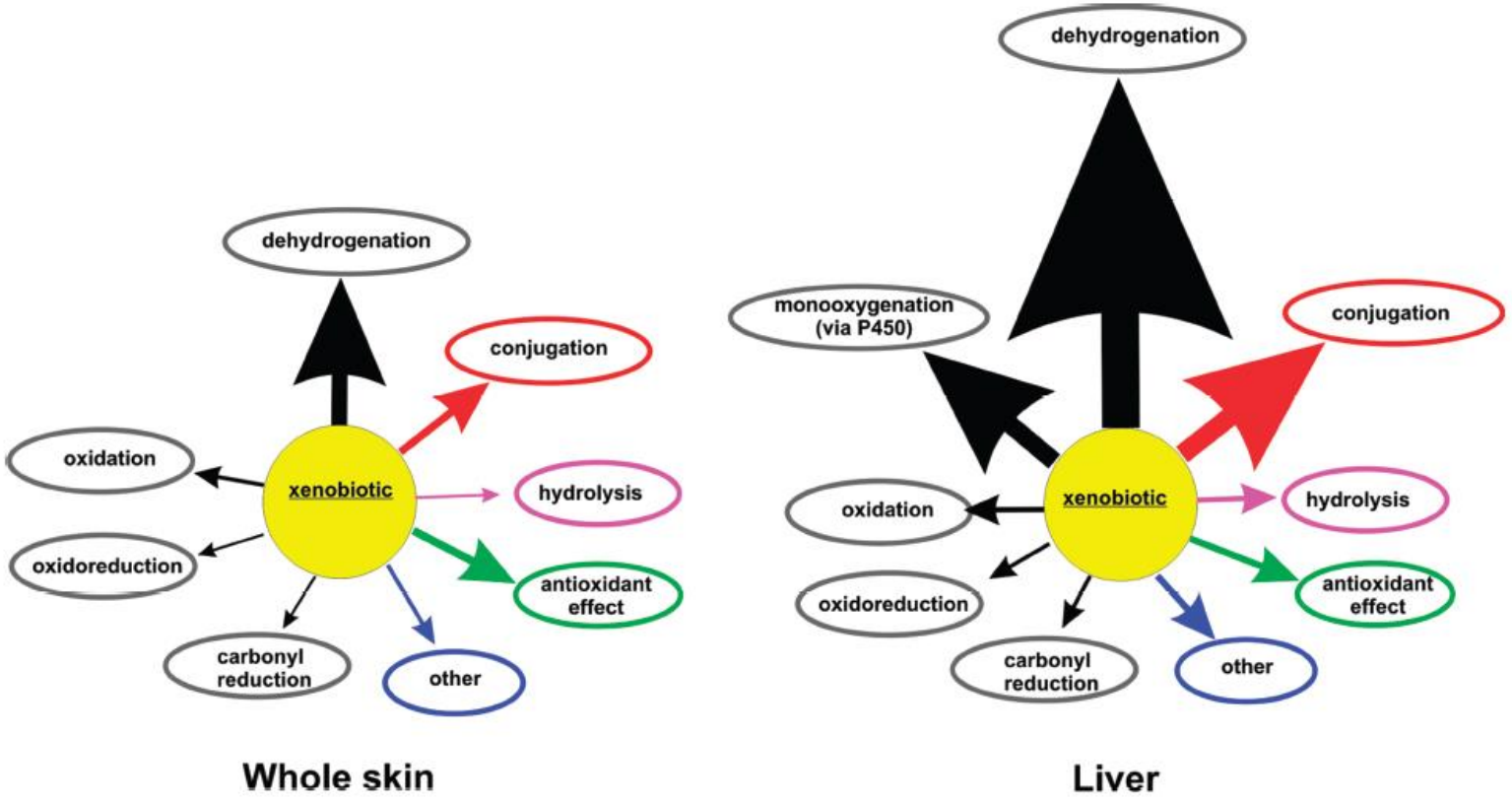
Skin Tissue-based Assays

Dermal application of test material, multiple application protocol enables enzyme induction



Hewitt et al, Metabolic competency of 3D skin models similar to human skin Toxicological Sciences 133(2), 209–217, 2013
Wiegand et al., Dermal xenobiotic metabolism: a comparison between native human skin, four in vitro skin test systems and a liver system. Skin Pharmacol Physiol. 27(5):263-75.

Historical control data of standard protocol

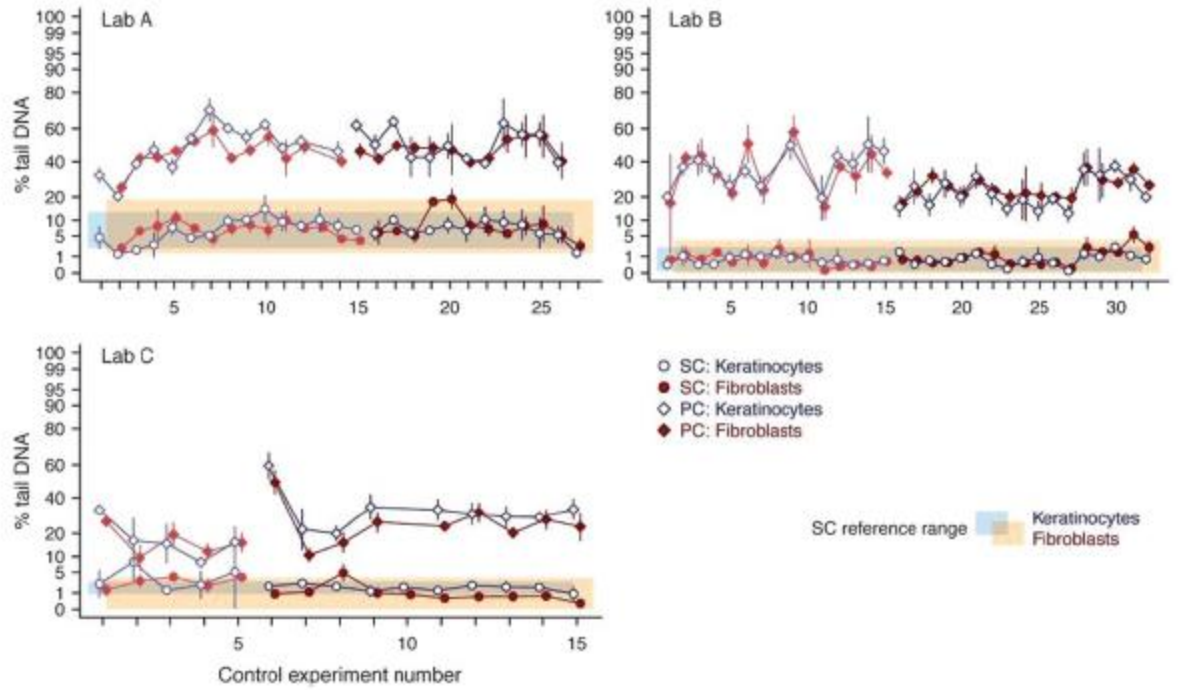


Figure 2. Historical control data of standard experiments. Percentage of tail DNA in the solvent control (SC) and positive control (PC, i.e., MMS) of individual experiments as obtained with the Phenion®FT during Phase 1 and 2 of coded testing are shown. The SC values (circles) and PC values (diamonds) for the keratinocytes (dark blue symbols and lines) and fibroblasts (red symbols and lines) are given as mean ± SD ($N = 3$ samples each). Faint symbols indicate values obtained in Phase 1 and dark symbols indicate values from Phase 2 of the validation. The light blue-shaded and orange-shaded areas indicate the reference range (mean ± 2 SD) for the SC, i.e. historical control, for keratinocytes and fibroblasts, respectively. The reference ranges were derived from the control data of Phase 2. The y-axis is on the arcsine square-root transformed scale, but the tick labels are expressed in units of the percentage scale.

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Historical control of APC experiments

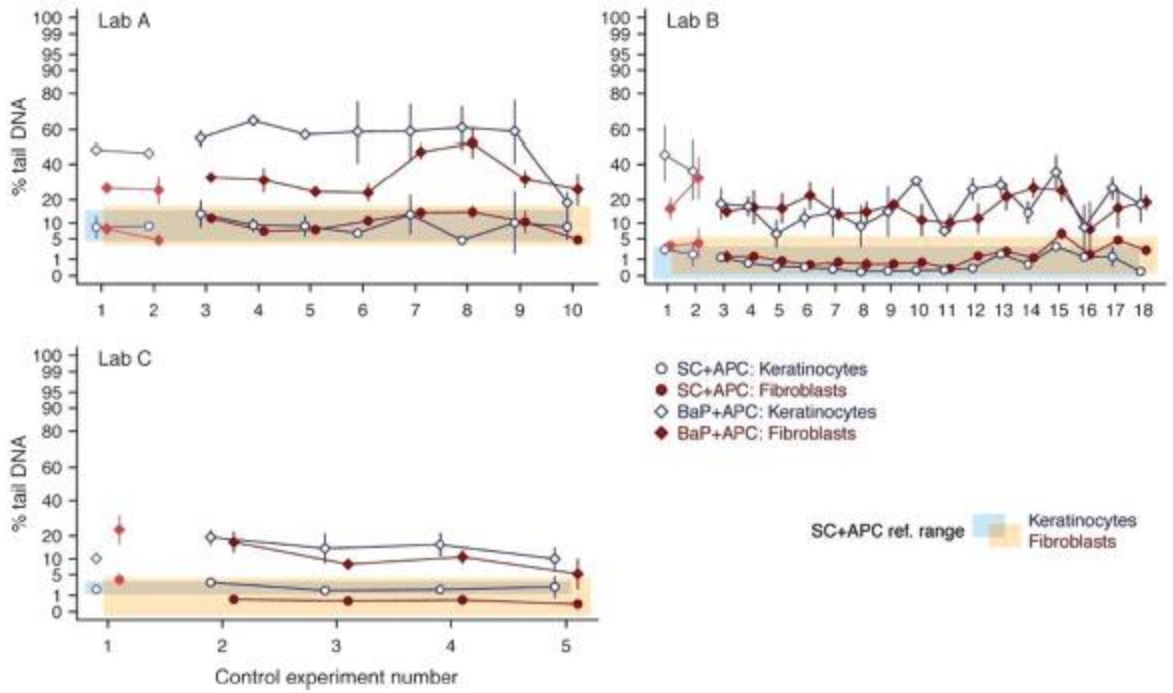

Fig. 3. Historical control data of APC experiments. Percentage of tail DNA in the solvent control (SC) and positive control (PC, i.e., BaP) both with APC of individual experiments as obtained with the Phenion®FT during Phases 1 and 2 of coded testing are shown. The SC + APC values (circles) and BaP + APC values (diamonds) for the keratinocytes (dark blue symbols and lines) and fibroblasts (red symbols and lines) are given as mean ± SD ($N = 3$ samples each). Faint symbols indicate values obtained in Phase 1 and dark symbols indicate values from Phase 2 of the validation. The light blue-shaded and orange-shaded areas indicate the reference range (mean ± 2 SD) for the SC, i.e., historical control, for keratinocytes and fibroblasts, respectively. The reference ranges were derived from the control data of Phase 2. The *y*-axis is on the arcsine square-root transformed scale, but the tick labels are expressed in units of the percentage scale.

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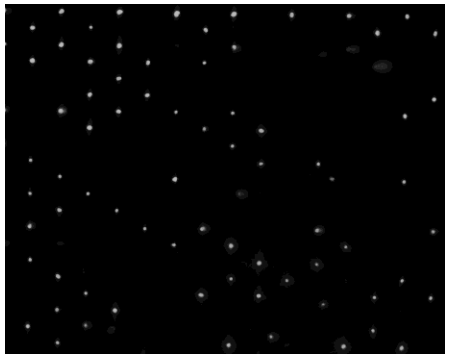
Improving scoring efficiency?

Slide by S. Pfuhler; work by Emily Rottinger, P&G

- Scoring of comet slides is time consuming, automated process could also improve data density
- Attempt to establish a protocol that enables the use of CometChip (CC) technology



Challenges to overcome:

- The skin comet assay utilizes Phenion FT tissues and a mincing protocol to generate a suspension of cells/nuclei
  - Cell number generated per tissue is rather low (lower than ideal for the CC), and somewhat inconsistent
  - The cells are not 'rounded', and don't load well into the CC

    -> Not enough cells for scoring
- Tried trypsin protocol which works well for RSMN. Loads better but generates high background for comet

References

Test protocol Validation phase I	Reisinger K, Blatz V, Brinkmann J, Downs TR, Fischer A, Henkler F, Hoffmann S, Krul C, Liebsch M, Luch A, Pirow R, Reus AA, Schulz M, Pfuhler S. Validation of the 3D Skin Comet assay using full thickness skin models: Transferability and reproducibility. Mutat Res Genet Toxicol Environ Mutagen. 827:27-41. PMID: 29502735
Validation Final evaluation	Pfuhler S, Pirow R, Downs TR, Haase A, Hewitt N, Luch A, Merkel M, Petrick C, Said A, Schäfer-Korting M, Reisinger K. Validation of the 3D reconstructed human skin comet assay, an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays. Mutagenesis. 36(1):19-35. doi: 10.1093/mutage/geaa009.
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Pre-project Analysis guidance	Reus AA, Reisinger K, Downs TR, Carr GJ, Zeller A, Corvi R, Krul CA, Pfuhler S. Comet assay in reconstructed 3D human epidermal skin models - investigation of intra- and inter-laboratory reproducibility with coded chemicals. Mutagenesis. 2013 28(6):709-20. PMID: 24150594
Case studies on hair dyes supported by the 3D Skin Comet assay and approved by the SCCS in the EU	B007-Basic Brown 17 http://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_152.pdf
	A138 -2,6-Dihydroxyethylaminotoluene http://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_188.pdf
	B34 -N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_196.pdf
Book chapters	In: Alternatives for dermal toxicology testing. Ed. C. Eskes. E. v. Vliet, H. Maibach, Springer Part V Genotoxicity 34. Pfuhler S, Reisinger K. Current Status. DOI 10.1007/978-3-319-50353-0_36
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	37. Reisinger K, Pfuhler S. Role in a Testing Strategy. DOI 10.1007/978-3-319-50353-0_39