Better Science, Better Ethics:
Animal-Free Cosmetic Safety Testing Approaches

RSC FTSG Personal Care Formulating

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Coming up…

1. XCellR8 and our mission
2. Truly animal-free testing – what is it and why does it matter?
3. Adapting a test to animal-free conditions (TG442d)
4. Developing an animal-free test (XtraMild)

Our talented team

- UK-based GLP accredited lab
- Expertise in ***in vitro*** testing for safety & efficacy
- Regulatory and non-regulatory testing
- Fully customisable methods
- The only 100% animal-product-free laboratory globally

Our mission

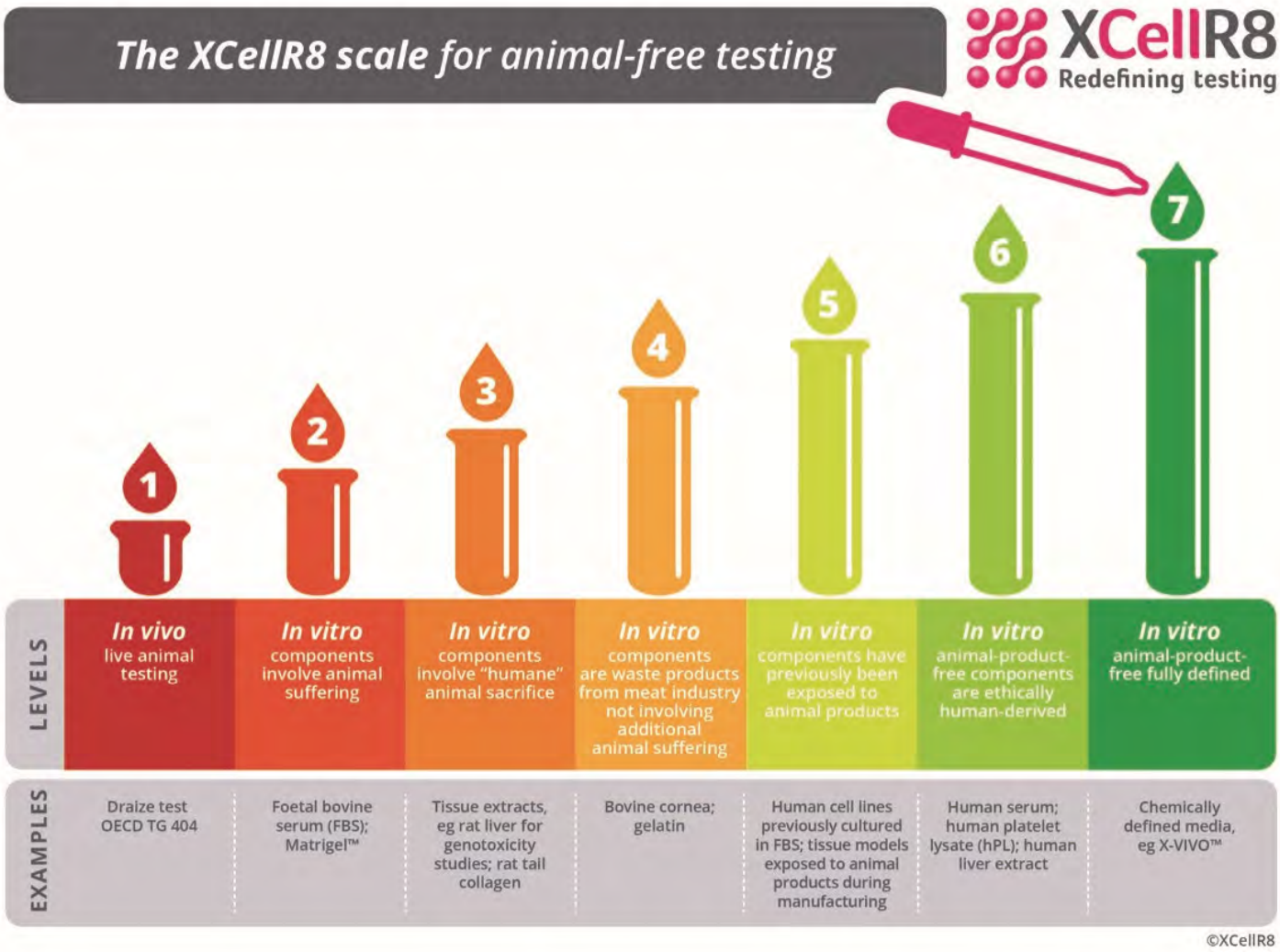
To accelerate the world's transition to **100% animal-free testing** through our scientifically advanced and ethical approach

Are cosmetics now tested without cruelty to animals?

- Ban in Europe fully in place since 11th March 2013
- 80% countries still test cosmetics on animals!
- Other regulations (e.g. REACH) require animal testing of ingredients in some cases.
  - Recent rulings by ECHA have put the ban at risk in the EU.
- Many “non-animal” tests still ultimately require animal sacrifice
- Ecotoxicology testing still requires animals, e.g. acute toxicity to fish

Truly animal-free testing

- Most ***in vitro*** methods use animal components
  - Foetal bovine serum
  - Tissue extracts
  - Antibodies
- Scientific and ethical considerations
- Improved reproducibility (when using chemically defined systems)
- Driven by consumer and industry demand for sustainable, ethical products (***and*** ethical testing)
- Truly animal-free testing needs to be animal-***p****roduct*-free
- Vegan products require vegan testing



Adaptation of an existing method:

KeratinoSens™
Skin Sensitisation Test (OECD TG 442d)
to Xeno-Free Conditions

Skin sensitisation – why testing is so important

- 1-3% (~7.5 million people) Europeans suffer from contact allergy to a cosmetic ingredient
- Skin sensitization to a cosmetic ingredient is a **permanent condition**
- ***In vitro*** tests provide an ethical alternative to human trials
- Preservatives and fragrances are the most common causes of skin sensitization in cosmetic products
- This is true for both natural and synthetic ingredients - **natural does not mean safe!**

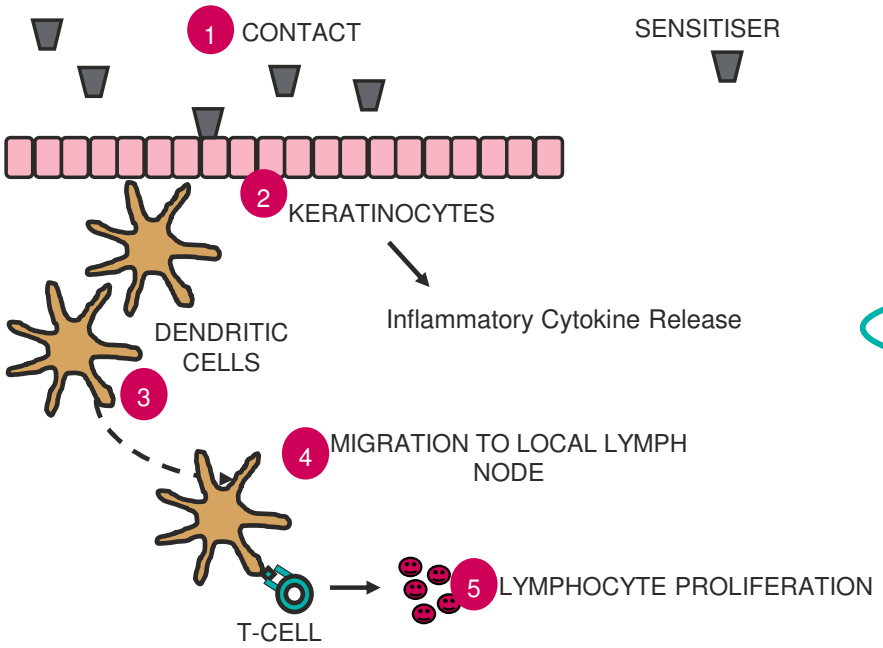
Animal-product-free skin sensitisation testing

THE CHALLENGE

Current regulatory guidance favours "2 out of 3" approach

- DPRA (OECD TG 442c)
- KeratinoSens™ (OECD TG 442d) **uses animal components**
- h-CLAT (OECD TG 442e) **uses animal components**

Skin sensitisation adverse outcome pathway (AOP)



KEY EVENTS IN SKIN SENSITISATION AND RELATED TESTS

1. Contact
   (Direct Peptide Reactivity Assay – **DPRA**)
2. Release of Pro-Inflammatory Cytokines by Keratinocytes (**KeratinoSens™**)
3. Dendritic Cell Activation/Maturation
   (human Cell Line Activation Test – **h-CLAT**)
4. Migration
5. T-cell Proliferation
   (Local Lymph Node Assay - LLNA)

Regulatory guidance: “2 out of 3” approach

Adaptation of the KeratinoSens™ Skin Sensitisation Test (OECD TG 442d) to Xeno-Free Conditions

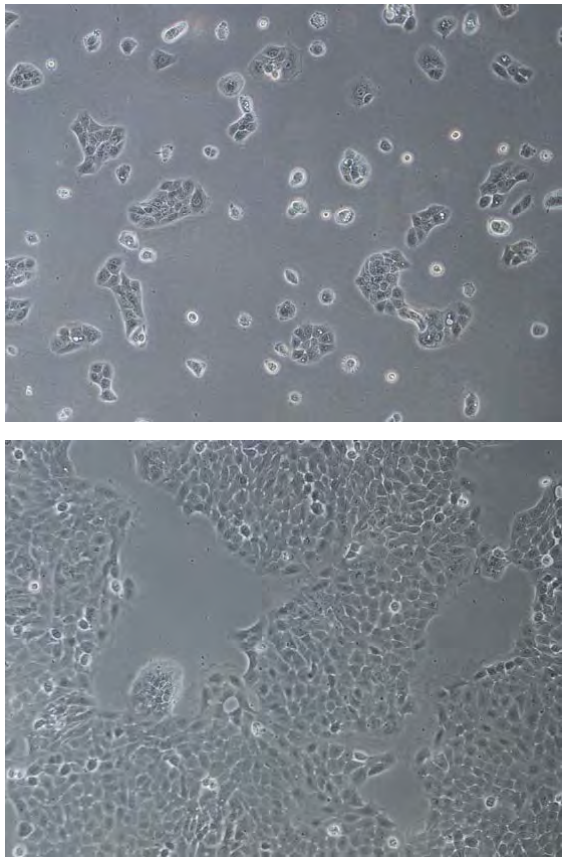
Published in ALTEX:

Belot, N., Sim, B., Longmore, CL., Roscoe, L. and Treasure, C. (2017)

Adaptation of the KeratinoSens™ skin sensitisation test to animal-product-free cell culture >

KeratinoSens™ - Method outline

- Human keratinocyte cell line (HaCaT) transfected with a luciferase reporter linked to Nrf2-mediated activation of Antioxidant Response Element (ARE)-linked genes
- 12 concentrations of test chemical incubated for 48 hours (in triplicate; 3 independent runs)
- Luciferase response measured by luminescence and cytotoxicity measured by MTT



Xeno-Free adaptation of KeratinoSens™

- Animal-derived components were replaced with human-derived & recombinant equivalents:
  - FBS replaced with pooled human serum (60-70 donors) obtained from FDA-approved source / Sigma Aldrich – cells adapted to new culture conditions
  - Porcine trypsin replaced with recombinant Trypzean™
- In-house validation using the panel of proficiency chemicals and performance standards for OECD TG 442d

Results: Non-Sensitisers (***as per LLNA***)

Chemical Name	Validated Reference Method (VRM)			XCellR8 Animal-Product-Free Adaptation		
	I_{Max}	EC1.5 (µM)	Prediction	I_{Max}	EC1.5 (µM)	Prediction
Isopropanol	1.2	n.i.	Non-Sensitiser	1.2	n.i.	Non-Sensitiser
Salicylic Acid	1.1	n.i.	Non-Sensitiser	1.4	n.i.	Non-Sensitiser
Lactic Acid	1.3	n.i.	Non-Sensitiser	1.3	n.i.	Non-Sensitiser
Glycerol	1.2	n.i.	Non-Sensitiser	1.4	n.i.	Non-Sensitiser
4-methoxy-acetophenone	1.7	449.3	***Sensitiser***	2.1	620	***Sensitiser***
Chlorobenzene	1.2	n.i.	Non-Sensitiser	1.2	n.i.	Non-Sensitiser
Methyl Salicylate	1.2	n.i.	Non-Sensitiser	1.2	n.i.	Non-Sensitiser
Sulfanilamide	1.4	n.i.	Non-Sensitiser	1.1	n.i.	Non-Sensitiser

n.i. = not induced

Results: Sensitisers (***as per LLNA***)

Chemical Name	Validated Reference Method (VRM)			XCellR8 Animal-Product-Free Adaptation		
	I_{Max}	EC1.5 (µM)	Prediction	I_{Max}	EC1.5 (µM)	Prediction
Cinnamyl alcohol	1.7	123.6	Sensitiser	4.2	20	Sensitiser
Ethylene Glycol Dimethacrylate	188	57.4	Sensitiser	4.8	29	Sensitiser
Phenyl Benzoate	1.3	n.i.	***Non-Sensitiser***	1.1	n.i.	***Non-Sensitiser***
Eugenol	1.3	n.i.	***Non-Sensitiser***	2.2	286	***Non-Sensitiser (borderline)***
2-Mercaptobenzothiazole	8.8	48.1	Sensitiser	6.9	57	Sensitiser
Citral	96.4	23.2	Sensitiser	3.8	18	Sensitiser
Isoeugenol	6.4	16.1	Sensitiser	3.4	20	Sensitiser
Methyldibromo Glutaronitrile	4	7.8	Sensitiser	2.7	8	Sensitiser
4-Methylaminophenol Sulfate	5.9	9.4	Sensitiser	36.1	4	Sensitiser
Para-phenylene Diamine	26.8	5	Sensitiser	28.2	6	Sensitiser
2,4-Dinitrochlorobenzene	14.8	2.5	Sensitiser	8.5	1	Sensitiser
4-Nitrobenzyl Bromide	6.9	1.3	Sensitiser	10.5	<0.98	Sensitiser
Oxazolone	2.4	175.5	Sensitiser	5.4	129	Sensitiser

n.i. = not induced

Animal-product-free (APF) adaptation of KeratinoSens™ Conclusions

- All 20 reference chemicals correctly classified in line with Validated Reference Method (VRM)
- Data accepted by the OECD Expert Working Group on Skin Sensitisation and WNT National Co-Ordinators' Committee
- Adapted method published as an Annex to the VRM in the new version of OECD TG 442d 2018
- Therefore full acceptance as a regulatory method

- Participation in thought-starter paper and OECD workshop on the ethical use of human reagents:
  - Addressing potential ethical issues regarding the supply of human-derived products or reagents in ***in vitro*** OECD Test Guidelines.

    Published in ALTEX 2019
- Xeno-free adaptation of h-CLAT including human serum and animal-free antibodies:
  - Edwards ***et al***, ALTEX, 2018
- Adaptation of KeratinoSens™ and h-CLAT to fully defined conditions

Creating an animal-free test:

XtraMild – Skin Mildness Test for Safety & Claim Support

Why we need a new method to predict mildness (I)

- Study of 12,377 individuals in Europe*
- Incidence of skin reactions lasting more than 3 days:
  - 19.3% within the last month
  - 31.8% within the last year
  - 51.7% within a lifetime
- Avoidance of daily life consumer products due to skin reactions:
  - 37.0% for skincare
  - 17.7% for “household or functional” products

* Naldi ***et al*** (2014). Prevalence of self-reported skin complaints and avoidance of common daily life consumer products in selected European regions. ***JAMA Dermatol 150(2): 154-162***

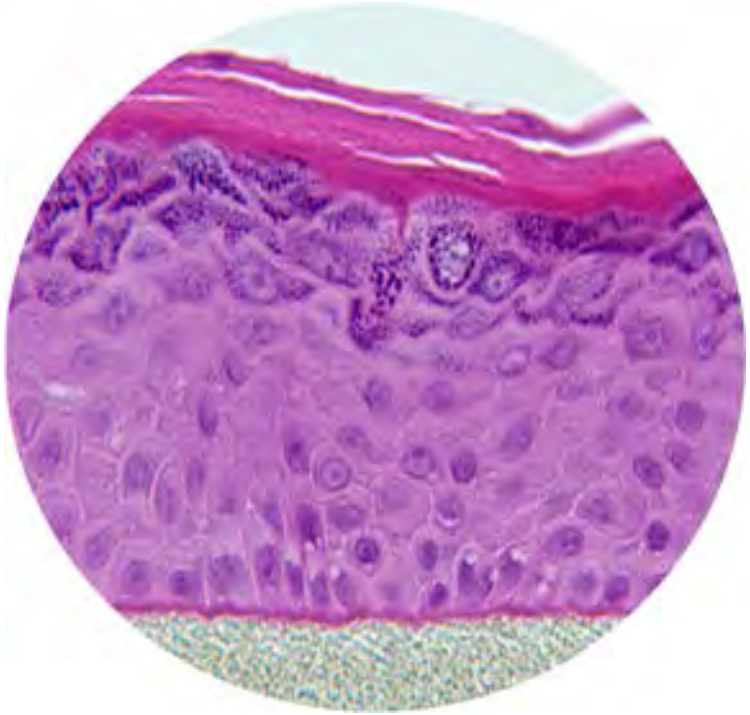
Why we need a new method to predict mildness (II)

- Increasing demand from consumers for ever milder products, that they feel confident using even when their skin is feeling extra sensitive
- Increasing demand from marketing teams for differentiating claims
- 2 year research project 2017-2019, funded by Innovate UK
- Research aims:
  - Optimising ***in vitro*** and human ***in vivo*** test methods for maximum sensitivity
  - Assess predictive capacity
  - Real world applications

We work with Innovate UK

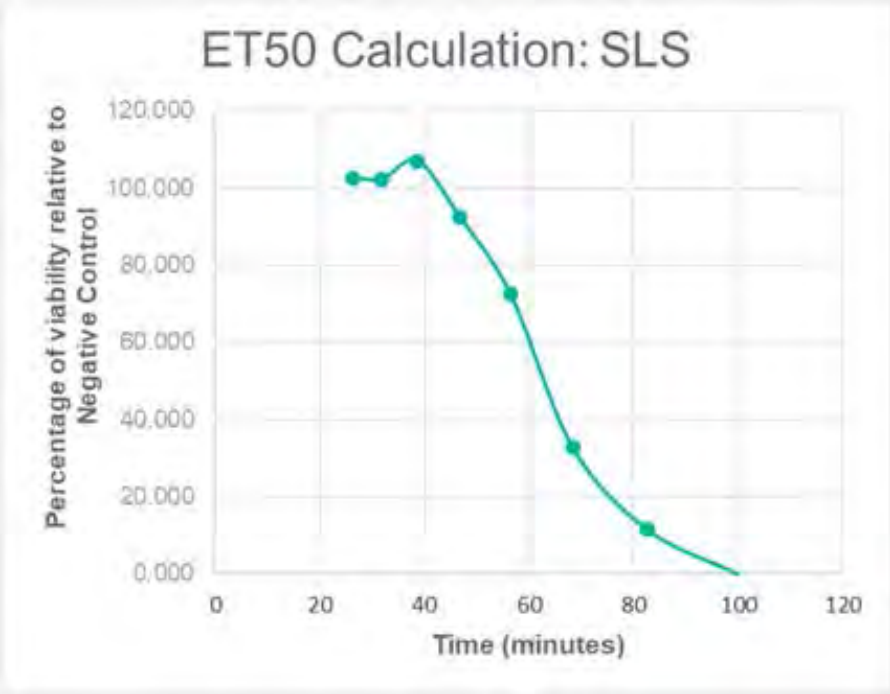
Existing methods: ***In vitro*** irritation testing

- 3D human skin models, grown at the air-liquid interface
- Suitable for testing ingredients and finished products
- Applied directly to the tissue surface – good model of "real life" exposure
- Standard regulatory method (OECD TG 439) measures a single exposure time to classify irritants vs non-irritants for hazard identification and labelling purposes
- Validated against historical animal data (Draize test)
- A more sensitive approach is required for today's mild cosmetic ingredients and formulations beyond a yes/no answer – ***how*** mild is the test item?



Cross section through reconstructed human epidermis

The ET50 method



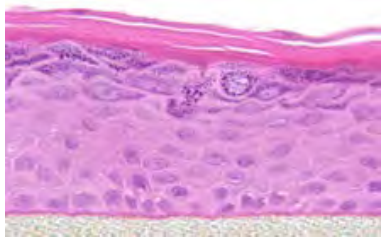
- Measures cell damage over a time course
- Classifies as Severe, Moderate, Mild or Minimal / Non-Irritant
- ET50 = time taken to reduce the viability of the skin model to 50% compared with untreated controls
- ET50 values allow rank order of irritation to be determined in comparison with other formulations / competitor and market leading products
- Standard methodology limited to 18 hours

How we optimised the test methods *in vitro*

- Development of an extended timepoint ***in vitro*** 3D model to look at the irritancy potential of ultra-mild test items over 48 hours
- Determination of ET_{50} values for known surfactant controls with a range of irritation potentials
- Development of a prediction model linking the ***in vitro*** skin irritation ET_{50} method with an ***in vivo*** human skin patch test model for ultra-mild surfactants
- Creation of a database of industry leading ingredients and formulations to be used as benchmarks in future tests for client companies

How we optimised the test methods *in vitro*

Test Items	Surfactants: SLS, SLES, CAPB, a novel “mild” surfactant
	Applied to the skin model surface and incubated for 1, 5, 18, 24 and 48 hours
Controls	Negative control: not treated
	Positive control: Triton X-100 (non-ionic surfactant): 1% solution
Measurement	Metabolic activity (conversion of MTT) as an indicator of cell damage
Output	ET50 value (time taken to reduce the viability of the cells to 50% compared with the untreated negative control)

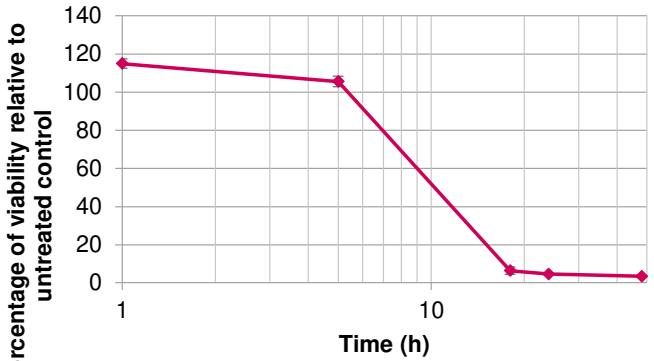


Determining the correlation between ***in vitro*** and ***in vivo*** results – some examples

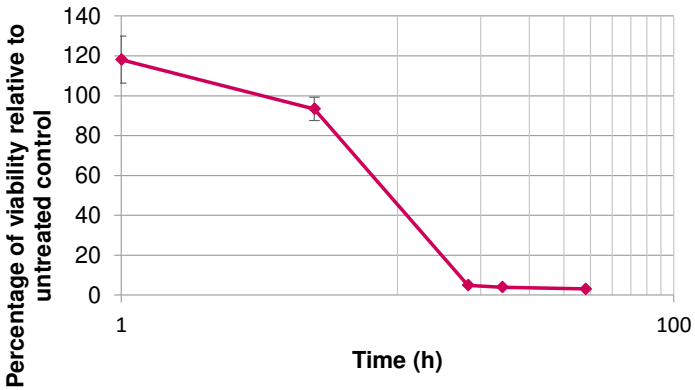
1. **SURFACTANTS**
2. **FACE MASKS**

In vitro irritation potential of 4 surfactants

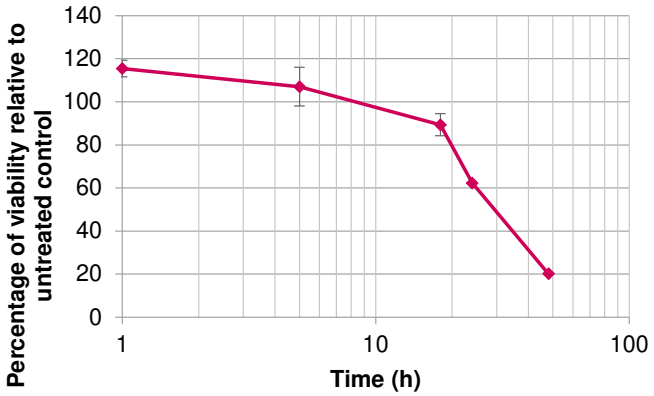
ET_{50} determination of A
0.3% SLES



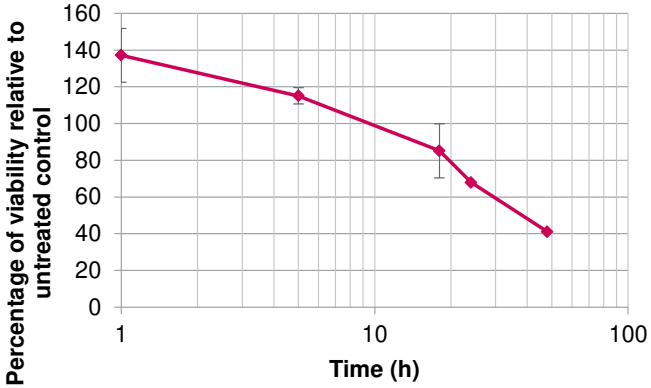
ET_{50} determination of C
0.3% SLS



ET_{50} determination of B
0.3% CAPB



ET_{50} determination of D
0.3% Novel surfactant



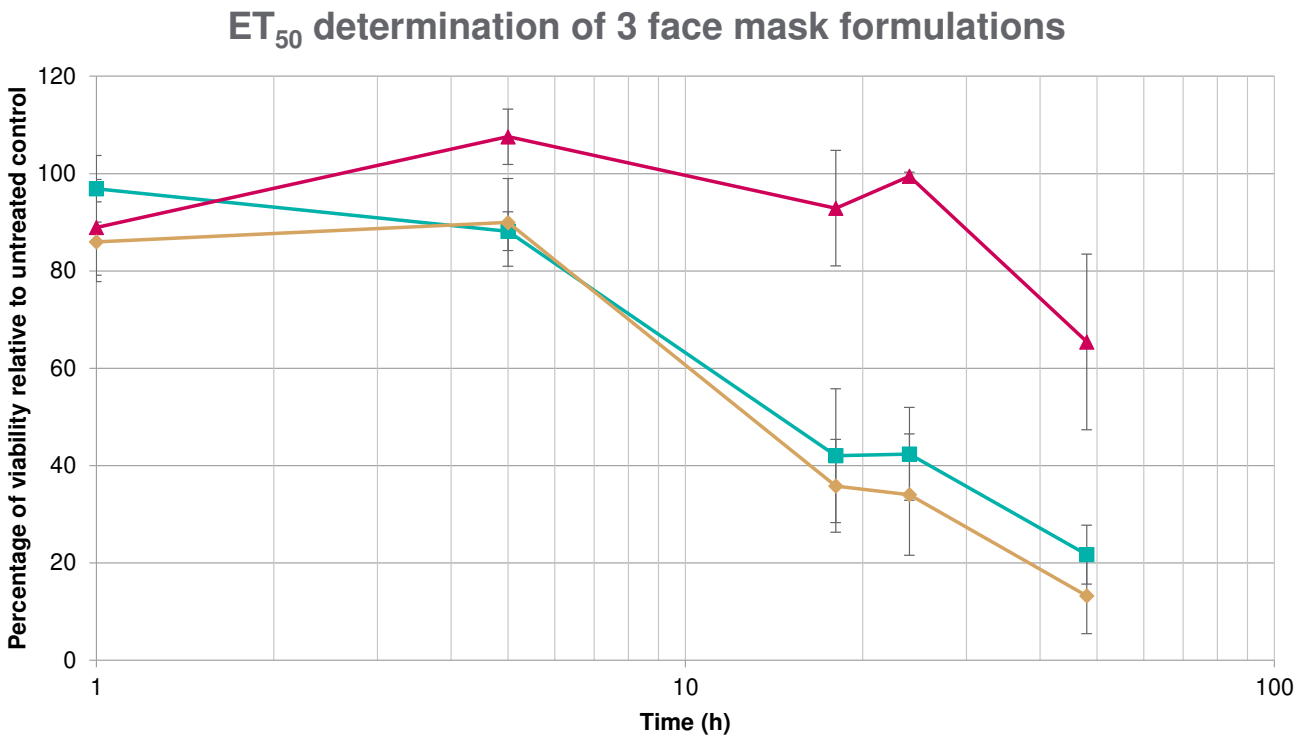
Test items (0.3%, pH 4.7) at
1, 5, 18, 24, 48hrs

Rank order of irritancy using linear extrapolation and logic equation

	C	>	A	>	B	>	D
ET50	9.37		10.25		29.4		38.08
CS	14		9		4		0

Irritancy classification:
C = SLS: Moderate to Mild
A = SLES: Moderate to Mild
B = CAPB: Non-Irritant
D = Novel surfactant: Non-Irritant

Face mask comparison *in vitro* and *in vivo*



Rank order of irritancy using linear extrapolation and logic equation

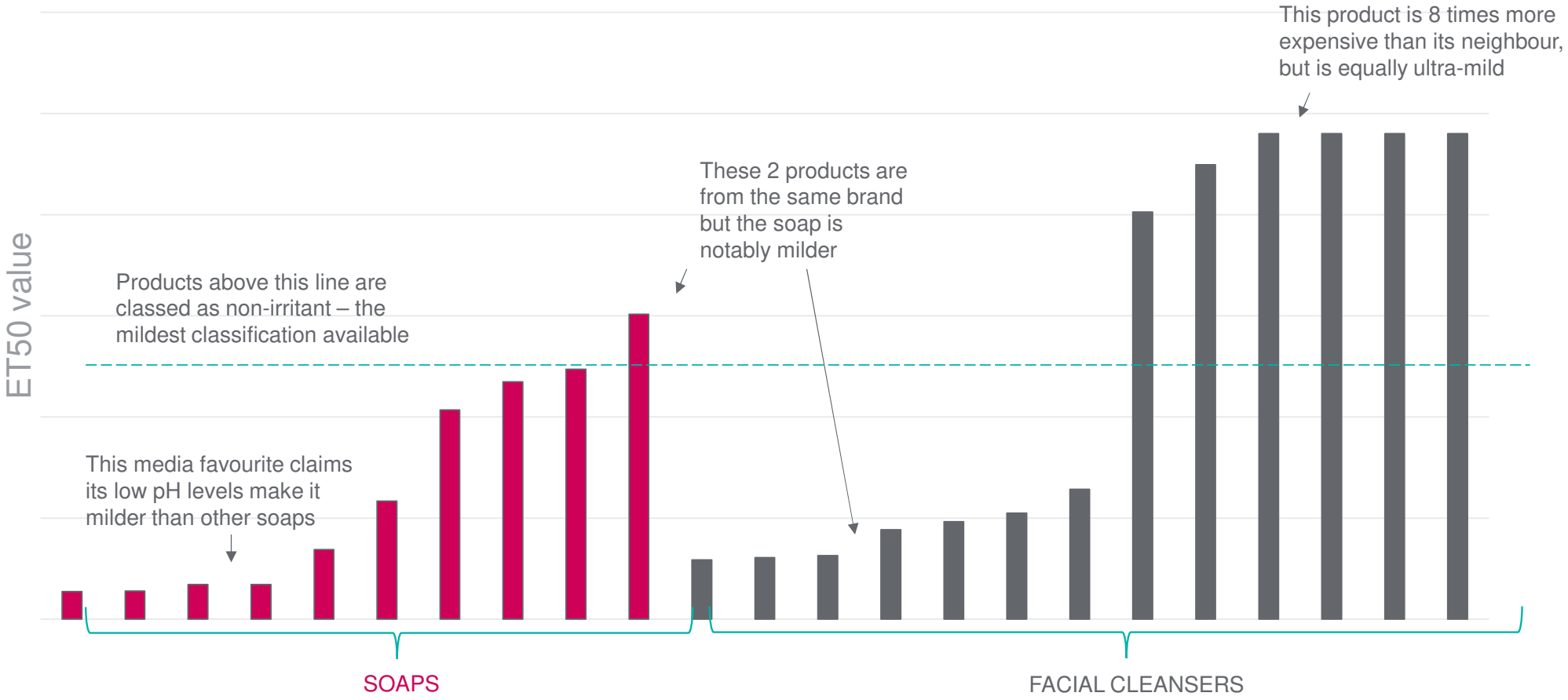
	B	>	A	>	C
ET50	12.86		14.42		>48
CS	11		5		2

IRRITANCY CLASSIFICATION

B = face mask 2: Very mild
A = face mask 1: Very mild
C = face mask 3: Non-irritating

Face mask C is the mildest product using this method

Building a reference database: relative mildness of soaps vs facial cleansers



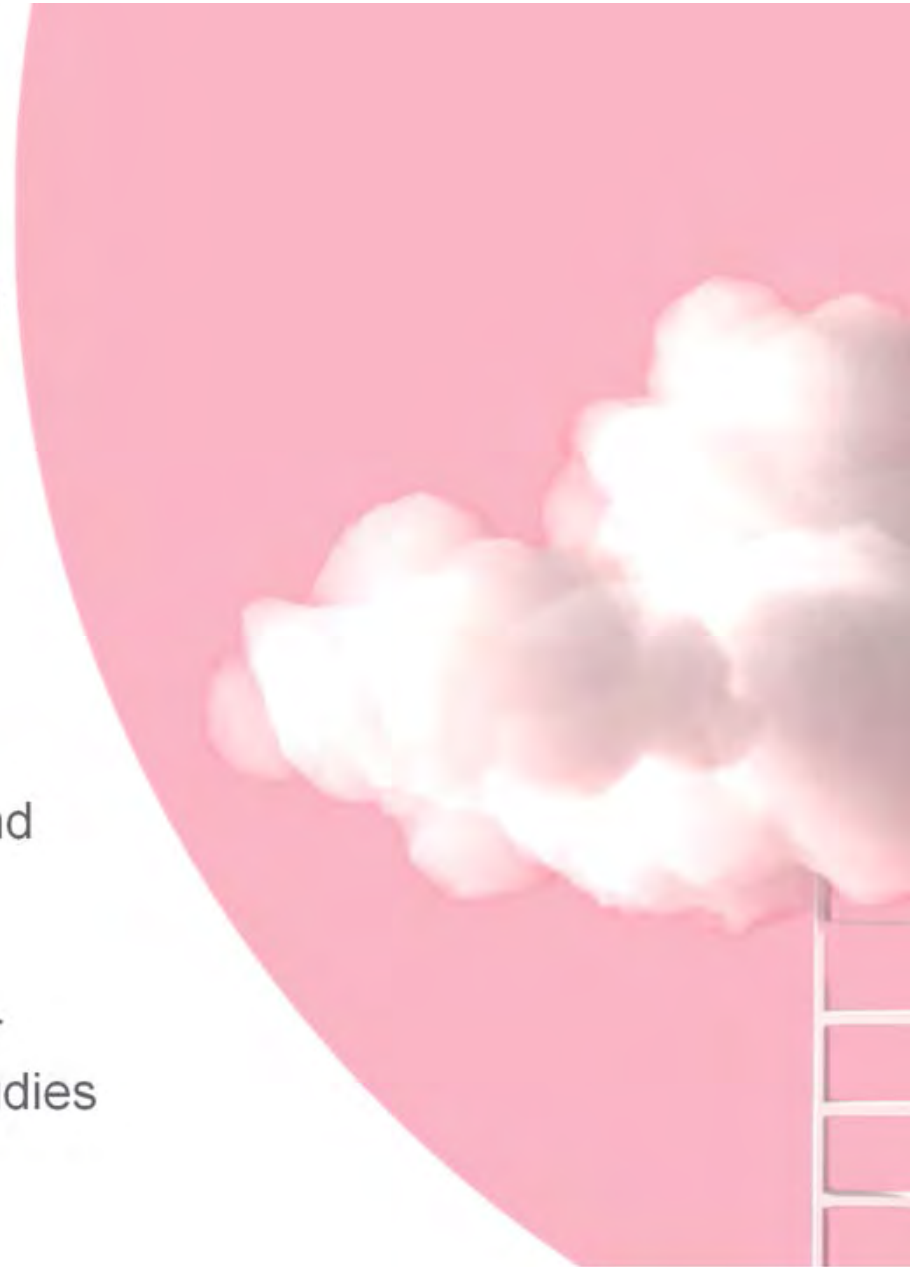
A variety of applications

- Ingredients:
  - Assessment of novel biosurfactants and other ingredients to assess mildness compared with other manufacturers and traditional materials
- Formulations:
  - ***In vitro*** benchmarking of new products against other brands or in-house formulations in development
  - Growing database for benchmark values currently includes:

✓ Facial soaps
✓ Facial cleansers
✓ Face masks
✓ Moisturisers
✓ Body soaps
✓ Shampoos
✓ Shower gels
✓ Sunscreens
✓ Deodorants
✓ Baby care products (oils, lotions, shampoos, bubble baths)

Take home…

- Many ‘non-animal’ tests still use animal components
- Using animal components raises scientific & ethical questions
- Adaptation of regulatory safety tests is already happening but it needs investment
- Technology and expertise to develop new animal product free methods is available
- ***In vitro*** testing provides robust safety information, and strong database for bench marking ingredients & formulations – IT’S BETTER SCIENCE
- Ethical advantages: limits human exposure, whether used as stand-alone test or pre-screen to clinical studies
- Marketing / consumer appeal



Thank you

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