XCellR8
Redefining testing

A new definition for animal-free testing. Replacing animal-derived components in regulatory *in vitro* methods.

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About XCellR8

- **Founded 2008** by Dr Carol Treasure and Bushra Sim

- UK-based CRO exclusively devoted to animal-free testing

Why?

Improve human safety without making compromises on *animal welfare*

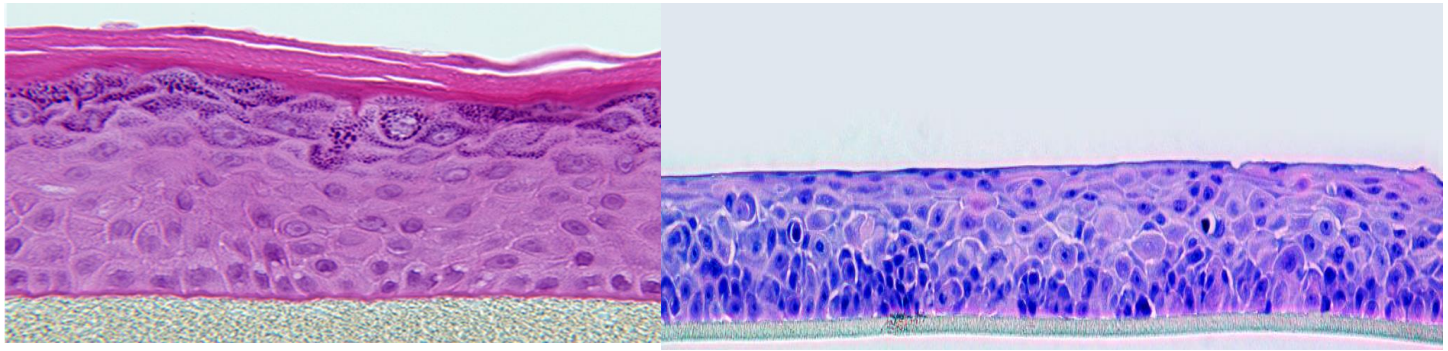
What is truly animal-free testing?

- Most *in vitro* methods utilise animal components:
  - Fetal bovine serum
  - Tissue extracts
  - Antibodies
- Reasons: largely historical
- Scientific and ethical considerations
- Truly animal-free testing needs to be animal-product-free
- Driven by consumer and industry demand for sustainable, ethical products *and* ethical testing

Everything we do at XCellR8 is animal-product-free (“vegan testing”) - GLP

Animal-Product-Free *In Vitro* Testing: Key Human Health Endpoints

- Skin Irritation (OECD TG 439): reconstructed skin model: **OK**
- Skin Corrosion (OECD TG 431): reconstructed skin model: **OK**
- Skin Corrosion: (OECD TG 435): Corrositex™: **OK**
- Eye Irritation (OECD TG 492): reconstructed corneal model: **OK**



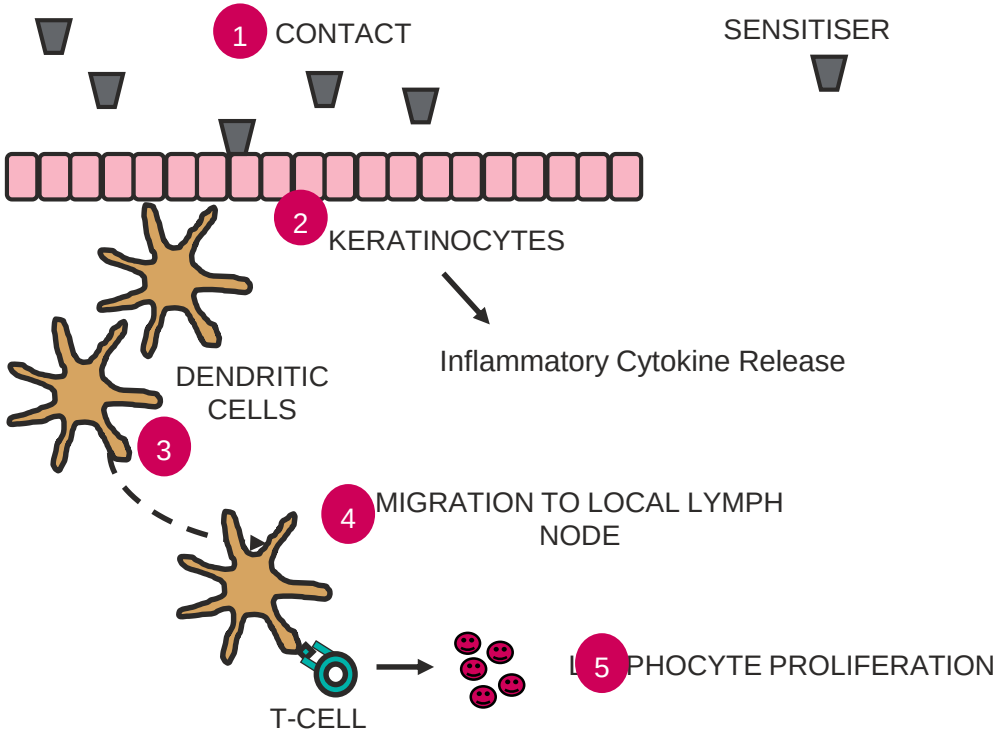
Animal-Product-Free *In Vitro* Testing: Key Human Health Endpoints

- Skin Sensitisation:
  - DPRA (OECD TG 442c): **OK**
  - KeratinoSens™ (OECD TG 442d): **animal components**
  - h-CLAT (OECD TG 442e): **animal components**

- Genotoxicity:
  - Ames Test (OECD TG 471): **animal components**
  - Micronucleus Test (OECD TG 487): **animal components**
  - Chromosome Aberration Test (OECD TG 473): **animal components**

- Acute toxicity: **no *in vitro* test** (LD50 related methods still in use)

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 N (Local Lymph Node Assay - LLNA)

Regulatory guidance: “2 out of 3” approach

Adaptation of the KeratinoSens™ Skin Sensitisation Test (OECD TG 442d) to Animal-Product-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. ALTEX.
http://www.altex.ch/current-issue/adaptation-of-the-keratinosens-skin-sensitization-test-to-animal-product-free-cell-culture

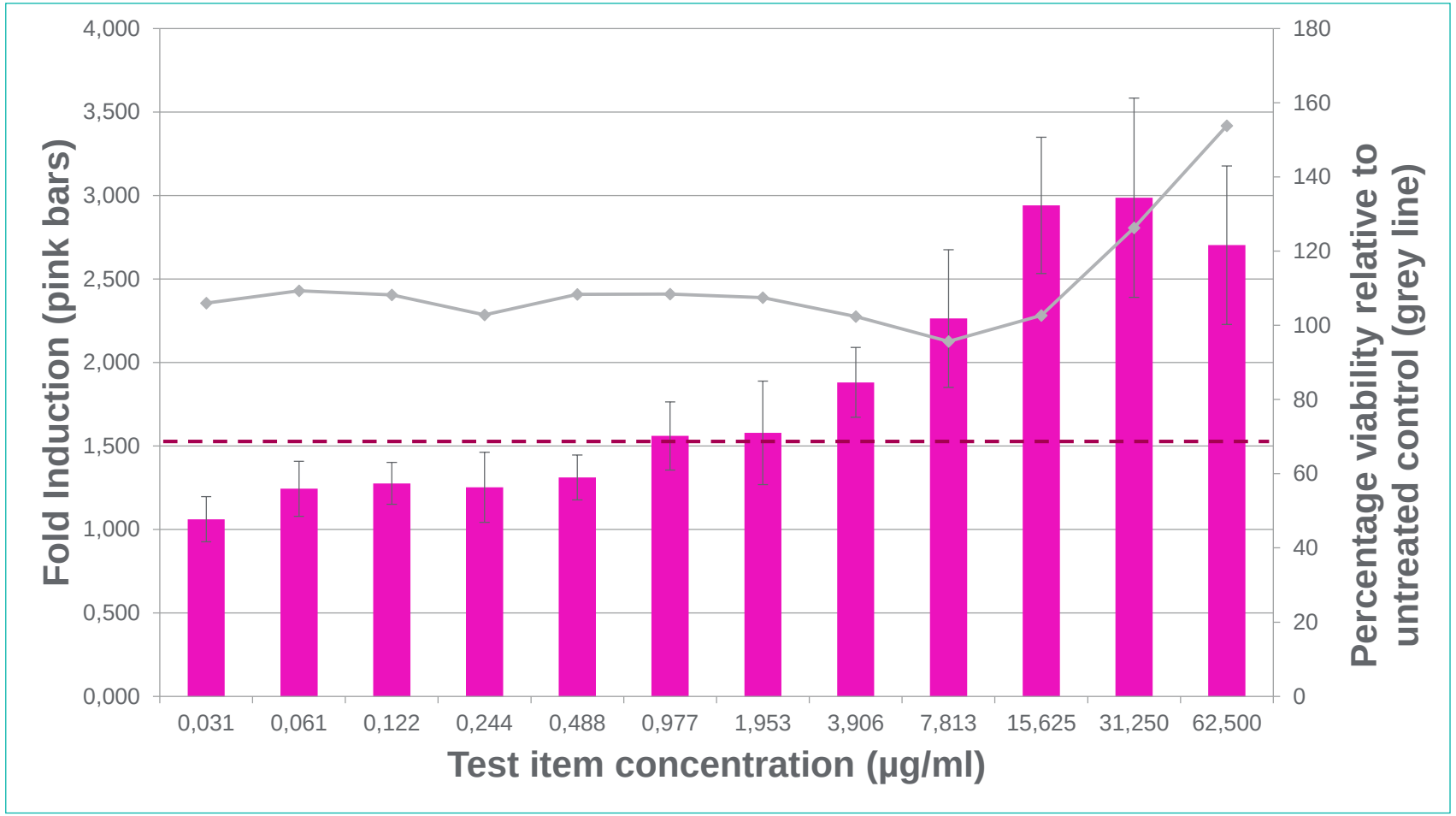
KeratinoSens™ - Method Outline

- 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

Animal-Product-Free (APF) 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.

KeratinoSens™ - Sample Results



Key parameters measured: EC1.5 (concentration to achieve 1.5-fold induction); Imax (maximum fold induction)

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 / Outcomes

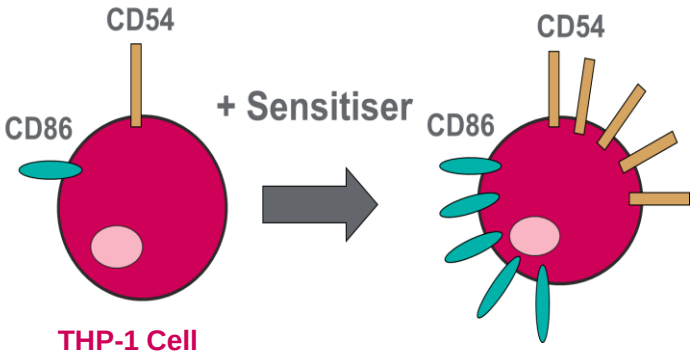
- All 20 reference chemicals correctly classified in line with Validated Reference Method (VRM)
- Data accepted by the OECD Expert Working Group on Skin Sensitisation and WNT National Co-Ordinators' Committee
- Adapted method published as an Annex to the VRM in the new version of OECD TG 442d 2018
-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

Adaptation of the human Cell Line Activation Test (h-CLAT) (OECD TG 442e) to Animal-Product-Free Conditions

Published in ALTEX:

Edwards *et al* (2018). Adaptation of the human Cell Line activation Test (h-CLAT) to Animal-Product-Free Conditions: https://www.altex.org/publib/Edwards_of_180613_v2.pdf

h-CLAT - Method Outline



- Measures changes in 2 dendritic cell surface markers (CD54 and CD86) – upregulated by skin sensitisers
- THP-1 cells (human monocytic cell line)
- Dose-finding assay to define CV75 (dose required to reduce cell viability to 75% of negative control values)
- Cells exposed to 8 concentrations of test chemical in at least 2 independent experiments
- Cells centrifuged, washed and treated with blocking buffer followed by FITC-conjugated antibodies for CD54 and CD86
- Analysis of Relative Fluorescence Intensity by flow cytometry. h-CLAT classifies sensitisation potential according to the crossing of a specific relative fluorescence intensity (RFI) threshold for each cell surface marker (RFI ≥ 200 for CD54; RFI ≥ 150 for CD86)

Animal-Product-Free Adaptation of h-CLAT

- *Animal-derived components were replaced with human-derived & recombinant equivalents:*
  - Fetal bovine serum (FBS) replaced with pooled human serum (60-70 donors) obtained from FDA-approved source / Sigma Aldrich – cells adapted to new culture conditions. *Validation performed in 3 independent batches*
  - Bovine serum albumin (BSA) replaced with human serum albumin (HSA)
  - Animal-derived anti-CD54 and anti-CD86 antibodies replaced with non-animal antibodies using phage display (Human Combinatorial Antibody Library: HuCAL) – optimised using *reactivity check* chemicals as defined in TG 442e (DNCB, Nickel Sulfate and Lactic Acid)

Animal-Product-Free Adaptation of h-CLAT

- *In-house validation* using the panel of proficiency chemicals and performance standards for OECD TG 442e, using 3 independent serum batches.

Results Part 1: CV75 Values

Proficiency Substance	TG 442E CV75 Value Reference Range (µg/ml)	XCellR8 Average CV75 Value (µg/ml)
2,4-Dinitrochlorobenzene	2-12	3.2
4-Phenylenediamine	5-95	32.6
Nickel sulphate	30-500	44.4
2-Mercaptobenzothiazole	30-400	276.4
R(+)-Limonene	>20	>500
Imidazolidinyl urea	25-100	14.2*
Isopropanol	>5000	>5000
Glycerol	>5000	>5000
Lactic acid	1500-5000	1546.4
4-Aminobenzoic acid	>1000	>1000

CV75 values (dose yielding 75% cell viability, µg/ml) derived from 2 independent experiments compared against the reference range from OECD TG 442E.

*Out of range. However TG 442e requires range to be met for 8 out of 10 substances

Results Part 2: CD54 and CD86 Regulation by Proficiency Substrates (Non-Sensitisers)

Proficiency Substance	XCellR8 EC200	XCellR8 EC150	VRM EC200 range	VRM EC150 range	Meets VRM Range?
Isopropanol	Neg (>5000)	Neg (>5000)	Neg (>5000)	Neg (>5000)	Yes
Glycerol	Neg (>5000)	Neg (>5000)	Neg (>5000)	Neg (>5000)	Yes
Lactic acid	*Neg (>1856)*	*Neg (>1856)*	*Neg (>5000)*	*Neg (>5000)*	*No**
4-Aminobenzoic acid	*Neg (>600)*	*Neg (>600)*	*Neg (>1000)*	*Neg (>1000)*	*No***

EC200 (CD54) and EC150 (CD86) values (Effective Concentration, µg/ml, at which the sensitisation threshold is crossed – at RFI values of 200 and 150 respectively) for each proficiency substance. Data shown are EC values derived from 2 independent concordant experiments.
*Due to the experimental CV75 derived (1546.4 µg/ml) the highest dose tested for Lactic Acid was 1856 µg/ml. **Due to solubility limitations the highest dose tested for 4-Aminobenzoic acid was 600 µg/ml.

Results Part 2: CD54 and CD86 Regulation by Proficiency Substrates (Sensitisers)

Proficiency Substance	XCellR8 EC200		XCellR8 EC150		VRM EC200 range	VRM EC150 range	Meets VRM Range?
2,4-dinitrochlorobenzene	1	5	1	4	0.5-15	0.5-10	Yes
4-Phenylenediamine	13	Neg	11	12	Neg or >1.5	<40	Yes
Nickel sulphate	48	45	40	44	10-100	<100	Yes
2-Mercaptobenzothiazole	103	89	63	93	10-140	Neg or >10	Yes
R(+)-Limonene	77	78	Neg	41	<250	Neg or >5	Yes
Imidazolidinyl urea	36	39	35	40	20-75	20-90	Yes

EC200 (CD54) and EC150 (CD86) values (Effective Concentration, µg/ml, at which the sensitisation threshold is crossed – at RFI values of 200 and 150 respectively) for each proficiency substance. Data shown are EC values derived from 2 independent concordant experiments.

Animal-Product-Free Adaptation of h-CLAT – Conclusions / Outcomes

- All proficiency chemicals correctly classified – equivalence with Validated Reference Method (VRM).
- Comparable values obtained for CV75, EC200 (CD54) and EC150 (CD86) (*see previous notes*)
- Data reviewed favourably by the OECD's WNT National Co-Ordinators' Committee and adaptation to TG442e will be discussed by Expert Working Group in 2018.
- Data may be used in REACH submissions supported by in-house validation results.

Further Work

- OECD discussions about animal-derived components in other methods and the use of human reagents

- Chemically defined conditions? To consider:
  - APF solutions driven by urgent need of key European cosmetic companies
  - Human derived components enabled a rapid solution to their key concerns
  - Human serum is from 60-70 pooled donors and good reproducibility observed between lots: for regulatory work, is this more representative than a chemically defined system?

eBook:

Getting under the skin of *in vitro* skin sensitisation testing

Available at:
https://x-cellr8.com/in-vitro-skin-sensitisation-testing/
Topics include potency assessment & testing finished products

Thanks to the XCellR8 team!











Thank you!

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