Skin sensitization testing strategy and in-house fit-for-purpose validations at Charles River Laboratories

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1 INTRODUCTION

The implementation of non-animal alternatives for skin sensitization testing has been driven by legislative changes, the 3Rs, and animal welfare considerations. The skin sensitization Adverse Outcome Pathway (AOP) testing comprises tests to address different key events in the AOP: peptide binding, antioxidant response element (ARE) mediated gene expression and dendritic cell activation.

The skin sensitization AOP is now specifically requested for REACH and EU Cosmetic Directive submissions but, due to the complexity of the AOP, no single *in vitro* test is capable of fully classifying substances into UN GHS categories. A number of tests are (or will soon be) accepted by OECD *via* EURL EVCAM. These tests are performed in a tiered testing strategy utilizing *in silico* (*i.e.* Derek), *in chemico* (*i.e.* DPRA, OECD 442C) and *in vitro* assays (including ARE-Nrf2 Luciferase Test, OECD 442D; h-CLAT, OECD 442E and U-SENS™, draft OECD test guideline). These tests are anticipated to almost completely replace *in vivo* tests such as LLNA (OECD 442A). Currently, for potency, REACH will almost certainly require LLNA.

Charles River Laboratories has performed fit-for-purpose validations of DPRA, ARE-Nrf2 Luciferase (KeratinoSens™, LuSens), h-CLAT and U-SENS™ tests using appropriate chemical proficiency panels. A testing strategy is also required to allow appropriate collation and interpretation of the results of the multiple tests. A proposed tiered strategy is presented.

2 METHODS

DPRA was performed according to OECD 442C (2015) OECD Guideline for the Testing of Chemicals: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA).

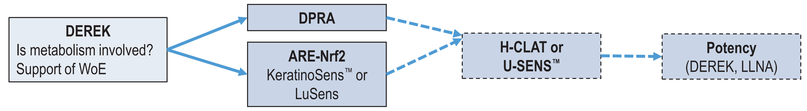
KeratinoSens™ assay was performed according to OECD 442D (2015) OECD Guideline for the Testing of Chemicals: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method.

LuSens assay was performed according to Protocol LuSens Assay (BASF, personal communication) and Ramirez *et al.* (2014) LuSens: A keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitisation hazard identification, Toxicology *in Vitro* 28; 1482–1497.

h-CLAT assay was performed according to OECD 442E (2016) OECD Guideline for the Testing of Chemicals: *In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT).

U-SENS™ assay was performed according to draft OECD guideline (2016) OECD Guideline for the Testing of Chemicals: *In Vitro* Skin Sensitisation: U937 Skin Sensitisation Test (U-SENS™).

3 Proposed 2 out of 3 Test Strategy for Mono-constituents (non-UVCB)



DPRA	ARE-Nrf2	Conclusion
Negative	Negative	**Non-Sensitizer:** Weight of Evidence approach
Positive	Positive	**Sensitiser:** Potency evaluation required
Positive	Negative	h-CLAT or U-SENS™ 2 out of 3 approach to determine sensitiser / non-sensitizer If sensitiser: further potency evaluation may be required
Negative	Positive	
Inconclusive*	Inconclusive*	

4 RESULTS

KeratinoSens™ Assay Test Panel and Results

Reference Chemical	*In Vivo* Classification	Experiment 1				Experiment 2				KeratinoSens™ Classification	Correct Classification?
		$EC_{1.5}$ (µM)	I_{max}	IC_{30} (µM)	IC_{50} (µM)	$EC_{1.5}$ (µM)	I_{max}	IC_{30} (µM)	IC_{50} (µM)		
2,4-Dinitro-chlorobenzene	Positive (Extreme)	1.64	11.6	6.96	8.34	1.59	14.0	6.04	15.54	Positive	Yes
4-Methylaminophenol sulfate	Positive (strong)	3.22	3.46	11.9	15.3	3.02	8.24	10.06	10.93	Positive	Yes
Methyldibromo glutaronitrile	Positive (strong)	18.8	1.71	26.1	33	4.22	4.93	15.5	17.5	Positive	Yes
2-Mercaptobenzothiazole	Positive (moderate)	1586	3.5	2353	>2400	371	2.43	689	1310	Positive	Yes
Cinnamyl alcohol	Positive (weak)	49.2	12.8	2195	>2310	14.9	6.95	1316	2013	Positive	Yes
Ethylene glycol dimethacrylate	Positive (weak)	38.9	137	579	793	22.7	56.5	548	724	Positive	Yes
Isopropanol	Negative	NA	1.37	NA	NA	NA	1.34	NA	NA	Negative	Yes
Salicylic acid	Negative	NA	1.41	NA	NA	NA	1.22	NA	NA	Negative	Yes
Lactic acid	Negative	NA	1.24	NA	NA	NA	0.97	NA	NA	Negative	Yes
Glycerol	Negative	NA	1.32	NA	NA	NA	1.06	NA	NA	Negative	Yes

LuSens Assay Test Panel and Results

Reference Chemical	*In vivo* Classification	Luciferase Induction		LuSens Classification	Correct Classification?
		Run 1[A]	Run 2[A]		
2,4-Dinitrochlorobenzene	Sensitiser (extreme)	3.08 ± 0.53	2.40 ± 0.12	Positive	Yes
4-Methylaminophenol sulphate	Sensitiser (strong)	3.25 ± 0.45	3.16 ± 0.80	Positive	Yes
Methyldibromo glutaronitrile	Sensitiser (strong)	3.08 ± 0.26	2.29 ± 0.73	Positive	Yes
2-Mercaptobenzothiazole	Sensitiser (moderate)	8.25 ± 0.93	10.94 ± 0.84	Positive	Yes
Cinnamyl alcohol	Sensitiser (weak)	8.11 ± 2.02	7.84 ± 1.30	Positive	Yes
Ethyleneglycol dimethylacrylate	Sensitiser (weak)	56.21 ± 1.17	33.93 ± 3.29	Positive	Yes
Glycerol	Non-sensitiser	0.84 ± 0.11	0.83 ± 0.29	Negative	Yes
Salicylic acid	Non-sensitiser	0.59 ± 0.07	0.71 ± 0.11	Negative	Yes
DL-Lactic Acid	Non-sensitiser	1.02 ± 0.22	0.89 ± 0.08	Negative	Yes
Isopropanol	Non-sensitiser	0.86 ± 0.07	0.51 ± 0.15	Negative	Yes

U-Sens™ Assay Results

CRL Data for Prospective Multicentre Study.
Sensitivity 94.7% (18/19)
Specificity 94.7% (18/19)
Accuracy 94.7% (36/38)

Alépée *et al.* (2015)
Piroird *et al.* (2015)

DPRA Assay Test Panel and Results

Reference Chemical	*In vivo* Classification	Reactivity Class			DPRA Classification	Correct Classification?
		Run 1	Run 2	Run 3		
p-Benzoquinone	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
2,4-Dinitrochlorobenzene	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
Oxazolone	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
Formaldehyde	Sensitiser (strong)	Moderate	Moderate	Moderate	Sensitiser	Yes
2-Phenylpropionaldehyde	Sensitiser (moderate)	Moderate	High	High	Sensitiser	Yes
Diethyl Maleate	Sensitiser (moderate)	High	High	High	Sensitiser	Yes
Benzylideneacetone	Sensitiser (moderate)	High	High	High	Sensitiser	Yes
Farnesal	Sensitiser (weak)	Moderate	Moderate	Moderate	Sensitiser	Yes
2,3-Butanedione	Sensitiser (weak)	High	High	High	Sensitiser	Yes
4-Allylanisol	Sensitiser (weak)	Moderate	Moderate	Moderate	Sensitiser	Yes
Hydroxycitronellal	Sensitiser (weak)	Low	Moderate	Low	Sensitiser	Yes
Butanol	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
6-Methylcoumarin	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
Lactic Acid	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
4-Methoxyacetophenone	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes

h-CLAT Assay Test Panel and Results

Reference Chemical	*In vivo* Classification	CD54 RFI[A]			CD86 RFI[A]			hCLAT Classification	Correct Classification?
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3		
2,4-Dinitrochlorobenzene	Sensitiser (Extreme)	921	281	N/R	194	256	N/R	Positive	Yes
4-Phenylenediamine	Sensitiser (Strong)	500	500	N/R	245	177	N/R	Positive	Yes
Nickel sulfate	Sensitiser (Moderate)	1956	1443	N/R	232	192	N/R	Positive	Yes
2-Mercaptobenzothiazole	Sensitiser (Moderate)	526	296	N/R	122	338	N/R	Positive	Yes
R(+)-Limonene	Sensitiser (Weak)	129	271	N/R	178	156	N/R	Positive	Yes
Imidazolidinyl Urea	Sensitiser (Weak)	2506	386	N/R	361	130	N/R	Positive	Yes
Isopropanol	Non-Sensitiser	152	36	31	171	97	62	Negative	Yes
Glycerol	Non-Sensitiser	88	47	N/R	85	85	N/R	Negative	Yes
Lactic acid	Non-Sensitiser	78	192	N/R	192	114	N/R	Negative	Yes
4-Aminobenzoic acid	Non-Sensitiser	139	32	N/R	99	47	N/R	Negative	Yes

5 CONCLUSIONS

Charles River Laboratories has demonstrated technical proficiency in DPRA, ARE-Nrf2 Luciferase tests, U-SENS™ and h-CLAT. Fit-for-purpose validations correctly assigning the skin sensitization potential of the chemical proficiency panels have been conducted. DPRA correctly assigned 12 sensitizers and 4 non-sensitizers. ARE-Nrf2 Luciferase (KeratinoSens™ and LuSens) tests both correctly assigned 6 sensitizers and 4 non-sensitizers. h-CLAT correctly assigned 6 weak to extreme sensitizers and 4 non-sensitizers. U-SENS™ was tested on a larger panel of 38 chemicals resulting in 98% sensitivity, 90% specificity and 94% accuracy.

In conclusion, a strategic battery of 3 tests, along with an appropriate *in silico* prediction model (e.g. Derek) combined with a weight of evidence approach, such as the 2 out of 3 model proposed by Bauch *et al.* (2012) and outlined in this poster allows for sensitive and appropriate predictions to be made. Inconclusive results may require to be clarified by LLNA, if appropriate. Potency assessment, as required, may be DEREK-based or by utilising LLNA testing.

References
Alépée *et al.* (2015) Toxicology *In Vitro* 30; 373 -382
Piroird *et al.* (2015) Toxicology *In Vitro* 29; 901-916
Bauch *et al.* (2012). Regul Toxicol Pharmacol;63(3); 489-504

Key
A: Values from highest non-toxic dose
N/R: Not Required,
WoE: Weight of Evidence
UVCB Substance of Unknown or Variable composition, complex reaction products or biological materials