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

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1 INTRODUCTION

The implementation of non-animal alternatives for skin sensitisation testing has been driven by legislative changes, the 3Rs, and animal welfare considerations. Skin sensitisation 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.

AOP-based testing 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, performed in a tiered testing strategy utilizing *in silico* (*e.g.* Derek), *in chemico* (DPRA, OECD 442C) and *in vitro* assays (including ARE-Nrf2 Luciferase Test, OECD 442D; h-CLAT, OECD 442E; U-SENS™, draft OECD guideline). These are anticipated to essentially replace in vivo tests such as LLNA (OECD 442A).

Charles River Laboratories has undertaken in- house fit-for-purpose validations for DPRA, ARE-Nrf2 Luciferase tests (KeratinoSens™, LuSens), h-CLAT and U-SENS™, using the appropriate chemical proficiency panels.

A testing strategy is required to allow appropriate collation and interpretation of the results of the multiple tests; a proposed tiered strategy is presented.

2 METHODS

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

The 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.

The 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).

The 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.

The 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 RESULTS

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

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 participated in a prospective multicentre study.
Sensitivity 94.74% (18/19)
Specificity 94.74% (18/19)
Accuracy 94.74% (36/38)

References:
Alépée *et al.* (2015) Toxicology In Vitro 30; 373 -382
Piroird *et al.* (2015) Toxicology In Vitro 29; 901-916

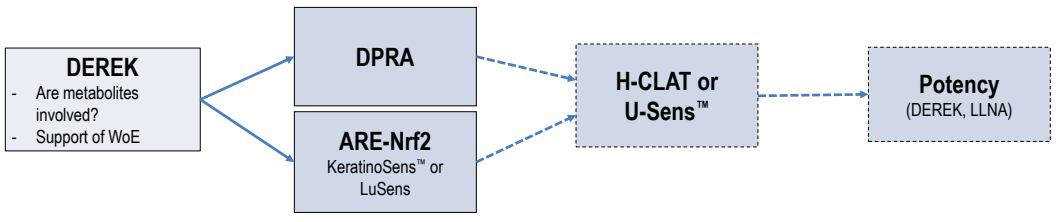
KeratinoSens™ Assay Test Panel and Results

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

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

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



DPRA	ARE-Nrf2	Conclusion
Negative	Negative	**Non-Sensitiser:** WoE approach
Positive	Positive	**Sensitiser:** Potency evaluation required
Positive	Negative	h-CLAT or U-Sens™ 2 out of 3 approach to determine sensitiser / non-sensitiser
Negative	Positive	
Equivocal*	Equivocal*	If sensitiser: further potency evaluation may be required

* If one or both tests gives equivocal results

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 sensitisation potential of the chemical proficiency panels have been conducted. The DPRA test correctly assigned 12 sensitisers and 4 non-sensitisers. The ARE-Nrf2 Luciferase (KeratinoSens™ and LuSens) tests correctly assigned 6 sensitisers and 4 non-sensitisers. The h-CLAT assay correctly assigned 6 weak to extreme sensitisers and 4 non-sensitisers. U-SENS™ was tested on a panel of 38 chemicals resulting in 95% sensitivity, 95% specificity and 95% accuracy.

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

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

Bauch *et al.* (2012). Regul Toxicol Pharmacol;63(3); 489-504