In Vitro Skin Sensitization – the 2 out of 3 Approach

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Skin Sensitization

- Allergic contact dermatitis is the clinical manifestation of a skin sensitization
- Hypersensitive reaction after repeated contact to an allergen
- 15 - 20% of the population sensitized
- Most common allergic contact dermatitis: nickel contact dermatitis
## Information requirements under REACH 2017: Update of Point 8.3 of Annex VII

<table>
<thead>
<tr>
<th>8.3.1. Skin sensitisation, <em>in vitro/in chemico</em></th>
<th>The(s) test(s) do not need to be conducted if:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Information from <em>in vitro/in chemico</em> test method(s) recognised according to Article 13(3), addressing each of the following key events of skin sensitisation:</td>
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<td>- molecular interaction with skin proteins;</td>
<td>- an <em>in vivo</em> study according to point 8.3.2 is available, or</td>
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<td>- inflammatory response in keratinocytes;</td>
<td>- the available <em>in vitro/in chemico</em> test methods are not applicable for the substance or are not adequate for classification and risk assessment according to point 8.3.</td>
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<td>- activation of dendritic cells.</td>
<td>If information from test method(s) addressing one or two of the key events in column 1 already allows classification and risk assessment according to point 8.3, studies addressing the other key event(s) need not be conducted.</td>
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</tbody>
</table>

| 8.3.2. Skin sensitisation, *in vivo* | An *in vivo* study shall be conducted only if *in vitro/in chemico* test methods described under point 8.3.1 are not applicable, or the results obtained from those studies are not adequate for classification and risk assessment according to point 8.3. |
The Skin Sensitization Mechanism

Stratum corneum

Epidermis

Hapten

Carrier protein

Keratinocyte

Dendritic cell ("Langerhans cell")

Courtesy of D. Urbisch
The Skin Sensitization Mechanism: KE1 (MIE) + KE2

Courtesy of D. Urbisch
The Skin Sensitization Mechanism: KE3

Courtesy of D. Urbisch
The Adverse Outcome Pathway for Skin Sensitization

The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins; Part 1: Scientific Evidence Series on Testing and Assessment No.168 ENV/JM/MONO(2012)10/PART1
OECD Adopted Test Guidelines

Test No. 442C: In Chemico Skin Sensitisation
Direct Peptide Reactivity Assay (DPRA)

Test No. 442D: In Vitro Skin Sensitisation
ARE-Nrf2 Luciferase Test Method

Test No. 442E: In Vitro Skin Sensitisation
In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation
Direct Peptide Reactivity Assay (OECD TG 442C)

- In chemico assay addressing the MIE of the skin sensitization AOP, i.e. protein reactivity
- Quantifies the reaction of a chemical with synthetic peptides containing cysteine (Ac-RFAACAA-COOH) or lysine (Ac-RFAAKAA-COOH)
- Chemical reactivity is expressed as peptide % depletion
- Mean of % C- and K- peptide depletion values used to discriminate between negative and positive results
Direct Peptide Reactivity Assay (OECD TG 442C)

- Not applicable for the testing of metal compounds

- Test chemicals that are not soluble at 100 mM may still be tested at lower soluble concentrations, BUT no firm conclusion on the lack of reactivity should be drawn from a negative result

- Technically applicable to the testing of mixtures of known composition (use of apparent molecular weight)

- According to the TG the current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances)

- Polymers are tested according to their predominant molecular weight or molecular weight of monomer; in addition tested undiluted
  - Test also undiluted / max. concentration of test substance

- Method does not encompass a metabolic system but majority of pre-haptens and pro-haptens are sufficiently well identified
Cell-based assays addressing the second key event in skin sensitization AOP, i.e. keratinocyte activation

Uses immortalised adherent cell lines (KeratinoSens™, LuSens) derived from human keratinocytes stably harbouring a luciferase reporter gene under the control of the antioxidant response element (ARE)
Applicable to test chemicals **soluble** or that form a **stable dispersion** either in water or DMSO (no longer a LogP limitation with the 2018 revision of TG 442D)

If above does not apply up to 2000 µM a negative result should be considered as inconclusive

May underpredict test chemicals exclusive reactive towards **lysine** residues

Limited **metabolic capability** but majority of pre-haptens and pro-haptens are sufficiently well identified

**Chemical stressors** may lead to false positive

Test chemicals (e.g. phytoestrogens) **interfering with the luciferase** enzyme and hence luminescence determination

Substances acting as **acylating agents** may be under-predicted
Cell-based assay addressing third key event of the skin sensitization AOP

Human cell line activation test (h-CLAT): Quantification of changes in the expression of cell surface markers associated with the process of activation of monocytes and DC (i.e. CD86 and CD54) in the human monocytic leukaemia cell line THP-1

FACS analysis of CD86 & CD54

CD54 (ICAM-1) → DC migration and T cell activation
CD86 (B7-2) → Co-stimulation during T cell activation
Test Methods Addressing Activation of Dendritic Cells (OECD TG 442E)

- Use only! THP-1 cells from ATCC (TIB-202™)
- Negative results for test substances with log $K_{OW} > 3.5$ and no cytotoxicity discussed in the ongoing revision are interpreted as “inconclusive. However, a positive result will be accepted.
- Limited information on multi-constituent substances/mixtures is available but test is technically applicable
- Applicable to test chemicals soluble or that form a stable dispersion
- Limited **metabolic capability** of the cell line but majority of pre-haptens and pro-haptens are sufficiently well identified
- **Fluorescent** substances interfering with the flow cytometric detection
OECD *In Vitro* Methods – metabolic capacity

- Approximately 25% of sensitizing substances are pre- or pro-haptens
- Great majority are pre-haptens
- Pre-haptens are generally correctly predicted by *in vitro* methods
- Slow oxidisers may not be correctly predicted, as in *in vivo* methods
- <10% of skin sensitizers are exclusively pro-haptens
  - Not identified by the DPRA
  - Correctly predicted by cell-based assays, with h-CLAT detecting the majority
- >90% of pre- and pro-haptens are correctly predicted by *in vitro* methods

Patlewicz et al., 2016
OECD Adopted Methods

- Detailed protocols available e.g. at: EURL ECVAM DB-ALM (ecvam-dbalm.jrc.ec.europa.eu)
- The Test Guidelines provide positive or negative predictions within the defined domain of applicability of an assay
- Negative predictions cannot be used on their own to conclude on the absence of skin sensitization potential of chemicals
- Although the test guidelines provide some quantitative information this cannot be used in isolation for the purpose of sub-categorisation (GHS Cat 1A and 1B)
- Data should be "considered in the context of Integrated Approaches to Testing and Assessment (IATA)", i.e. in combination with complementary information
REACH Guidance on IR&CSA

The *in vitro* tests for which OECD TG are available can – and must – be used for the assessment of the skin sensitisation potential!

The animal test is, however, still needed when:

- *in vitro* are not applicable
  (lipophilic or highly cytotoxic substances, mixtures, ...)

- *in vitro* results are ambiguous
  (discordant single test results, pro-haptens, ...)

More than 50% of all substances?
OECD TGs – Use Under REACH

Start here

PART 1:
Retrieving existing information
(Skin sensitisation testing and assessment strategy: Elements 1-5)*

Sufficient for C&L including potency and risk assessment, if needed?

yes

HAZARD INFORMATION*
Consider for classification including potency assessment, labelling and risk assessment, if needed.

no

PART 2:
Weight-of-Evidence judgement
(Skin sensitisation testing and assessment strategy: Element 6)

Sufficient for C&L including potency and risk assessment, if needed?

yes

HAZARD INFORMATION
Consider for classification including potency assessment, labelling and risk assessment, if needed.

no

PART 3:
Generation of new testing data* (Skin sensitisation testing and assessment strategy: Elements 7-9)

Assessment largely based on weight-of-evidence
A Defined Approach consists of a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources (formalised decision-making approach).
OECD Guidance Documents (GD) on Defined Approaches

Six defining principles:
1. Defined endpoint
2. Defined purpose
3. Description of the underlying rationale, including mechanistic basis (e.g. AOP)
4. Description of the individual information sources used
5. Description of how the individual information sources are processed
6. Consideration of the known uncertainties

GD 255 Templates for reporting
GD 256 Case studies
## Defined Approaches – Case Studies

<table>
<thead>
<tr>
<th>Case Study</th>
<th>Bioavailability</th>
<th>Phys-chem properties</th>
<th>in silico</th>
<th>Protein binding/reactivity</th>
<th>Events in Keratinocytes</th>
<th>Events in DC</th>
<th>Events in T cells</th>
<th>Adverse effect</th>
<th>Others</th>
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</thead>
<tbody>
<tr>
<td>1 Sensitiser potency prediction Key event 1+2 (Givaudan)</td>
<td>X</td>
<td>TIMES SS</td>
<td>Cor1C420-assay</td>
<td>TG 442D</td>
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<td>2 The artificial neural network model for predicting LLNA EC3 (Shiseido)</td>
<td>X</td>
<td>SH Test</td>
<td>AREc32 assay</td>
<td>TG 442E</td>
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<td>3 ITS/DS for hazard and potency identification of skin sensitisers (P&amp;G)</td>
<td>penetration (PBPK model)</td>
<td>TIMES SS</td>
<td>TG 442C</td>
<td>TG 442D</td>
<td>TG 442E</td>
<td>US37 test</td>
<td>TG 429</td>
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<td>4 Tiered system for predicting sensitising potential and potency of a substance (ITS) (Kao Corporation)</td>
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<td>TG 442C</td>
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<td>5 Score-based battery system for predicting sensitising potential and potency of a substance (ITS) (Kao Corporation)</td>
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<td>6 IATA for skin sensitisation risk assessment (Unilever)</td>
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<td>modified OECD TG418</td>
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<td>7 Weight of evidence in vitro ITS for skin hazard identification (BASF)</td>
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<td>modified OECD TG418</td>
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<td>8 STS for hazard identification of skin sensitisers (RIVM)</td>
<td>Various</td>
<td>TG 442C</td>
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<td>9 IATA (Dow)</td>
<td>X</td>
<td>Various</td>
<td>TG 442C glutathione depletion assay</td>
<td>TG 442D</td>
<td>TG 442E</td>
<td>U937</td>
<td>TG 429</td>
<td>TG 406</td>
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<td>10 Decision strategy (L’Oréal)</td>
<td>X</td>
<td>Various</td>
<td>TG 442C</td>
<td>TG 442D</td>
<td>ARIE-HirL2 Assay</td>
<td>U-SENS™ PGE2 Assay</td>
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<td>11 Integrated decision strategy for skin sensitisation hazard (ICCVAM)</td>
<td>X</td>
<td>OECD Toolbox</td>
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<td>12 Consensus decision tree model for skin sensitisation hazard prediction (EC IRC)</td>
<td>X</td>
<td>TIMES SS</td>
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Some based fully on *in vitro* methods, some on *in silico*, some combine both.

The *in vitro* methods are mainly OECD Test Guidelines, but some are not.

Algorithms used to combine data to make a prediction vary in complexity.

Annex 1 to Guidance Document No. 256
DA Case Study I: “2 out of 3“ for Hazard ID

The results of any 2 of the 3 tests determine the overall result (testing strategy) with very good predictivity (94%).

Baubch et al., 2012
Predictive Capacity of 2 out of 3 Approach

In vitro WoE Approach

- **Accuracy**: 79%
- **Sensitivity**: 82%
- **Specificity**: 72%

for comparison:
- DPRA: Accuracy 75%

Urbisch et al., 2015

Human data

- **n = 114**
- **73% Sens.**
- **27% non-Sens.**

In vitro WoE Approach

- **Accuracy**: 90%
- **Sensitivity**: 90%
- **Specificity**: 90%

for comparison:
- LLNA: Accuracy 82%
- DPRA: Accuracy 84%
The 2 out of 3 approach is an AOP based hazard identification DA providing mechanistic data.

The 2 out of 3 approach achieves slightly better predictivites than the LLNA compared to human data.

Technical limitations of individual test methods apply.
OECD Project on The Development of a TG on Defined Approaches for Skin Sensitisation

- Definition of an internationally agreed evaluation framework for DAs
- Translation of scientific valid DAs into a TG that would fall under MAD
- First draft **Guideline on Defined Approaches for Skin Sensitization** is available and open for comments until 16 Nov 2018
Summary

- Standard information requirement for REACH updated in the light of scientific progress. Potential to produce significant sensitization in humans has to be considered.

- Information on the first three key events of the AOP should be addressed in first place with the validated and OECD adopted methods and for test items shown to be in their domain of application.

- Methods adopted so far need to be used in combination to generate sufficient evidence for negative results and significant effects.

- In the near future it may be possible to have one-to-one replacements for the LLNA, so far it is not.

- DAs for skin sensitization appear promising for predicting LLNA and human responses.

- Ongoing OECD activities aim to give to DAs the same regulatory recognition as the animal tests.
Some References


BASF TRAINING

SKIN SENSITIZATION

Thursday 22\textsuperscript{nd} - Friday 23\textsuperscript{rd} of November 2018
Ludwigshafen, Germany