Getting under the skin of *in vitro* sensitisation testing
Dr Carol Treasure
Founder and CEO of animal-free testing lab, XCellR8

Carol has devoted her career to using innovative science to replace animal testing. She holds a PhD from the FRAME Alternatives Laboratory at the University of Nottingham, UK, and has worked in the field for over 25 years.

XCellR8 is the only contract testing laboratory globally to adopt a completely animal-product-free approach, avoiding the use of all animal-derived components in its regulatory and non-regulatory testing programme. The company has achieved regulatory recognition for its adaptations of in vitro regulatory tests.

Today, XCellR8 continues to innovate, using its scientifically advanced and ethical approach to accelerate the world’s transition to 100% animal-free testing.

In vitro skin sensitisation tests are now the default requirement for compliance with a variety of legislation such as the European REACH and CLP regulations.

Regulatory acceptance has paved the way for methods once known as “alternatives” to become the mainstream, and the use of traditional methods using the guinea pig and mouse are rapidly diminishing. For the cosmetics industry in Europe, and an increasing number of other regions, animal testing bans have fuelled investment into new, scientifically advanced approaches, and the field is constantly moving forward.

Only a few years ago, the availability of reliable in vitro tests for skin sensitisation seemed out of reach, partly because of our relatively poor understanding of the biology of sensitisation in the human body. An industry-wide, truly international collaborative approach led to a detailed definition of the Adverse Outcome Pathway (AOP), and a battery of in vitro tests to address the key endpoints soon followed. The tests were originally developed for hazard identification and labelling purposes and therefore focussed on a “yes/no” result (is a test chemical a skin sensitiser or not?)

THE NEXT GENERATION OF SKIN SENSITISATION TESTS

Now the debate is shifting to how we can modify our approaches to maximise the usefulness of these tests. For example, can they predict potency? Can they be used to test complex mixtures such as plant extracts and finished products?

As an industry, we continue to build on this momentum to drive forward new ways of utilising existing data and to create the next generation of animal-free tests. Keeping up with the fast-paced changes – and how they apply to the everyday reality of product safety assessment for individual businesses – can seem overwhelming at times, especially for SMEs (small and medium enterprises) where one person is expected to wear several hats. That’s why we decided to put this guide together; to summarise the key information, decode the jargon and clarify when in vitro sensitisation tests can be used. We’ve added an at-a-glance jargon buster on page 13 for quick reference. We hope the book is a useful and interesting read. Please do get in touch and let us know your thoughts – we’d love to hear from you.
Before we debate what the tests can be used for, let’s start with a brief look at the biology.

A skin sensitiser is a substance that will lead to an allergic response following skin contact (as defined by the United Nations Globally Harmonised System). Repeat exposure is a key feature of sensitisation, which is usually considered in two phases: the induction phase describes the initial physiological response to a chemical, creating a memory in the body’s immune system that will lead to an allergic reaction (the elicitation phase) next time the skin is exposed to the same chemical structure. Currently, all regulatory in vitro tests for skin sensitisation focus on the induction phase.

**WHY SKIN SENSITISATION TESTING IS IMPORTANT**

Since sensitisation is a complex pathway involving the immune response, it’s generally considered a much more serious skin reaction than irritation. Skin irritation is a short-term, local, reversible reaction in the skin. In contrast, once an individual is sensitised to a particular cleaning chemical or cosmetic product, they will typically be sensitised for life – so it’s an extremely important part of safety assessment.

**WHAT HAPPENS DURING THE SKIN SENSITISATION PATHWAY?**

By its very nature, the complex pathway of skin sensitisation was considered notoriously difficult to model in vitro: how could such an intricate, multi-step, physiological pathway be accurately represented in a single test? Thanks to the significant efforts of multiple scientific groups over more than a decade, we now have a portfolio of three in vitro skin sensitisation tests with direct relevance to humans.

The tests address the first three key events in the Adverse Outcome Pathway (AOP). In simple terms these are: the test chemical binding to skin proteins; skin cell activation; and immune cell activation.

We now have a portfolio of three in vitro skin sensitisation tests with direct relevance to humans.
The three regulatory *in vitro* tests for skin sensitisation explained

The complex pathway of skin sensitisation was only recently mapped out in detail, using a system known as an Adverse Outcome Pathway (AOP). This was a critical development as it paved the way for a whole animal test system to be replaced with a combination of mechanistic, human-based *in vitro* methods.

The three tests measure human responses to a chemical at three points in the AOP – these are described as key events.

- **The first key event is contact between the chemical and skin proteins.** Protein binding increases the likelihood of the chemical being a skin sensitiser. This is measured by the Direct Peptide Reactivity Assay (DPRA).
- **Key events 2 and 3 are measured using human cell culture based tests that assess the activation of skin epidermal cells (keratinocytes) and immune cells (dendritic cells) – these are the KeratinoSens™ and h-CLAT methods respectively.**

**KEY EVENTS IN SKIN SENSITISATION TESTS:**

1) CONTACT (direct peptide reactivity assay - DPRA) OECD TG 442C
2) RELEASE OF PRO-INFLAMMATORY CYTOKINES AND ACTIVATION OF KERATINOSES (KERATINOSENS™) OECD TG 442D
3) DENDRITIC CELL ACTIVATION/MATURATION (human cell line activation test - hCLAT) OECD TG 442E
4) T-CELL PROLIFERATION (local lymph node assay - LLNA) OECD TG 429 – ANIMAL TEST
When should I use *in vitro* skin sensitisation tests?

**CURRENT OECD GUIDANCE**

A variety of global regulations now cite the three *in vitro* tests as the default approach for skin sensitisation assessment, including the European Chemicals Agency (ECHA) Guidance on the applicability of the methods to REACH (we recommend this as a user-friendly read!)

As with any testing strategy, the known capabilities and limitations of the methods must be taken into account. Current OECD guidance is provided in each of the relevant Test Guidelines and we’ve summarised the key points in the table on the next page for quick reference.

Current regulatory guidance, including IATA (Integrated Approaches to Testing and Assessment) and the guide by ECHA, describes a “two out of three” approach, whereby two positive results would lead to classification of a chemical as a skin sensitiser. Ideally, all three tests should be performed to build the best possible picture of the human skin sensitisation potential of the chemical or mixture.

However, in practice, many labs are first performing two tests (usually the DPRA and KeratinoSens™), only following up with a third (h-CLAT) if confirmation is needed to obtain the two out of three classification. This strategy is often cost-driven as the h-CLAT is the most expensive of the three tests, and commercial factors drive resistance to replacing the traditional animal tests. While this remains the mainstream approach, there are some current views in favour of using the DPRA and h-CLAT combination (Roberts and Patlewicz, 2016), as well as a school of thought that the DPRA is the single most predictive test, since it looks at the molecular initiating event of the AOP (Benigni et al, 2016). Test chemicals should be considered on a case-by-case basis to take scientific and commercial factors into account, while prioritising a comprehensive safety assessment.

It’s worth keeping in mind that the *in vitro* tests were all originally validated against historical data from the Local Lymph Node Assay (LLNA). The table shows strong performance data for all three *in vitro* methods, using the LLNA as a benchmark. However, the LLNA has many drawbacks including a high incidence of false positive results, variation with dose vehicle and predictivity issues (eg inconsistencies with human patch test data) (Anderson et al, 2011). All three of the *in vitro* TGs state “Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA, as well as other animal tests, may not fully reflect the situation in the species of interest, i.e. humans.” The validation of *in vitro* methods against less-than-ideal benchmarks is an ongoing challenge, and more creative approaches to validation are needed.
## Summary of OECD guidance on the 3 test methods

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>DPRA OECD TG 442C</th>
<th>KeratinoSens™ OECD TG 442D</th>
<th>h-CLAT OECD TG 442E</th>
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<tbody>
<tr>
<td>SCOPE</td>
<td>Discrimination between sensitisers and non-sensitisers in the context of IATA - combined with data from <em>in vitro</em> tests assessing other events in the sensitisation pathway (AOP) as well as read-across from chemical analogues.</td>
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<tr>
<td>LIMITATIONS</td>
<td>May not be used as a stand-alone method to sub-categorise skin sensitisers into UN GHS 1A and 1B (high or low-to-moderate sensitisers respectively), or to predict potency (see below) for safety assessment decisions.</td>
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<tr>
<td>PERFORMANCE DATA</td>
<td>Accuracy: 80%  Sensitivity: 80%  Specificity: 77%</td>
<td>Accuracy: 77%  Sensitivity: 78%  Specificity: 76%</td>
<td>Accuracy: 85%  Sensitivity: 93%  Specificity: 66%</td>
</tr>
<tr>
<td>APPLICABLE TEST CHEMICALS</td>
<td>Validated and shown to be suitable for a wide variety of organic functional groups, reaction mechanisms, skin sensitisation potencies and physico-chemical properties.</td>
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<tr>
<td>RELEVANCE FOR MIXTURES</td>
<td>Can be used only for mixtures of known composition due to dependency of the prediction model on the molar ratio of the test item to the cysteine and lysine peptides. Additional testing of the neat mixture may allow confirmation of the result.</td>
<td>Can be used for mixtures. Components that are toxic to cells can skew the results. Parallel cytotoxicity measurement therefore essential.</td>
<td></td>
</tr>
<tr>
<td>SPECIFIC EXCLUSIONS</td>
<td>Metal compounds.</td>
<td>Chemicals that are highly toxic to cells; chemicals that interfere with the luciferase enzyme.</td>
<td>Strongly fluorescent chemicals may interfere with detection (alternative antibodies may be considered).</td>
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<tr>
<td>DOES IT CONSIDER METABOLISM?</td>
<td>Pro-haptens: no.  Pre-haptens: sometimes (<em>In chemico</em> method with no in-built metabolic system)</td>
<td>Pro-haptens and pre-haptens both under-predicted (The cell line used has limited metabolic capability).</td>
<td></td>
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<tr>
<td>CAN IT PREDICT POTENCY?</td>
<td>Potentially contribute to the assessment of potency when used in integrated approaches such as IATA. There is a demand for further work based on human data.</td>
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<tr>
<td>SOLUBILITY LIMITATIONS</td>
<td>Must be soluble in an appropriate solvent at 100mM.</td>
<td>Must form a solution or stable suspension in water or DMSO. Links to LogP* values as follows: Log P &lt;5: compatible. Log P 5-7: limited information. LogP &gt;7: outside known applicability.</td>
<td>Must form a solution or stable suspension in a suitable solvent, preferably saline, cell culture medium or DMSO. LogP* &gt;3.5 can lead to false negative results but can be used to support positive classification.</td>
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</table>

**The Log P value, or octanol/water partition coefficient (Kow), is defined as the ratio of a chemical’s concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system. It is therefore a measure of the aqueous solubility of the test chemical and its compatibility with the test method.**
Assessing potency

The three *in vitro* tests are validated and approved for hazard identification and labelling purposes.

This means that, when taken together with other sources of information, such as computer modelling (*in silico*), they enable a “yes / no” classification – is the chemical a skin sensitisier (UN GHS Class 1) or not? It’s important to recognise that they are not currently formally validated to predict potency (how sensitising is the chemical?) As such, the methods can’t be used to distinguish between the UN GHS sub-categories 1A (high sensitisers) and 1B (low to moderate sensitisers), or to rank sensitising chemicals according to potency. The Test Guidelines state that the methods may potentially contribute to the assessment of potency when used in integrated approaches such as IATA. There is a demand for further work based on human data to support this work.

To some extent, the DPRA is the most informative of the three methods because the percentage depletion of peptides results in a classification as a Minimal, Low, Moderate or High Sensitiser. In the KeratinoSens™ method, the EC1.5 value (the concentration required to cause a 1.5-fold induction of the cellular response) holds the potential to be used as supporting information.

A comprehensive dataset (145 chemicals) produced from both the DPRA and KeratinoSens™ tests showed that analysis of the dose-response parameters of each test indicated a correlation to sensitisation potency (**Natsch et al., 2013**). Taken together, this information shows us that the tests do provide some useful potency data in their current form when building a weight of evidence, but the information cannot be relied upon in a stand-alone context.
POTENCY CONTINUED

There are several current initiatives to modify existing in vitro tests to improve potency assessment, as well as the development of new methods that take this important factor into account. One such method is GARDskin™ developed by Senzagen in Sweden. GARDskin™ is a genomic test measuring changes in the expression of 200 genes relevant to the skin sensitisation AOP. The method is currently under validation in the OECD Test Guidelines programme and includes GARDpotency™, an add-on to the standard method which is able to sub-categorise sensitisers as class 1A or 1B according to the UN GHS system, by monitoring the expression of 52 complementary genomic biomarkers. The adoption of genomic technology into skin sensitisation testing is an exciting development with great potential for the future.

... A WORD ABOUT METABOLISM

No guide to skin sensitisation testing would be complete without a word on metabolism! This can be a big factor in the sensitisation profile of many chemicals, since metabolism may significantly change their reactivity. Pro-haptens are chemicals that require conversion by enzymes to exert their sensitisation potential, whereas pre-haptens can convert to metabolites without any biological enzyme system. As the TGs point out, the three validated methods have limitations: the KeratinoSens™ and h-CLAT methods use cell lines with only limited metabolic capability and the DPRA is an in chemico method (biochemical reaction) with no cells present. Currently the TGs suggest that, while pro-haptens cannot be detected by these methods, some pre-haptens can be correctly classified. Some recent research by Patlewicz et al (2016) looked at an ECVAM dataset of 127 chemicals, concluding that sensitisers requiring activation were identified correctly using one or more of the current in vitro tests. In all cases, the overall safety assessment of chemicals classified as non-sensitisers needs to take account of these limitations, and the test results combined with other data within the framework of an IATA to provide as much information as possible.

How to GARD™ your product in six steps

1. **GARD Input Finder**
   - Grown SenzaCells

2. **GARD Main Stimulation**
   - Add different concentrations of the test substance to the cells

3. **RNA extraction**
   - Take test substance at determined concentration and add to fresh batch of cells

4. **Gene expression profiling**
   - Determine the concentration of the test substance where the cells react and 98% survive
   - Mix the isolated RNA with reporter probes and load onto a cassette
   - Analyze the probe cassette to quantify the RNA

5. **GARD data analysis application**
   - Upload the results to the GDAA web app
   - One press of the button and the algorithm crunches the data

6. **Results**
   - The results are yours!
Relevance for mixtures

The three *in vitro* skin sensitisation tests (and the LLNA) were validated using a panel of individual reference chemicals.

However, many cosmetic companies need to assess the safety of final formulations, to meet their responsibilities under the European Cosmetic Regulation 1223/2009, which includes an animal testing ban. Other industry sectors including agrochemicals and household products also have to ensure the safety of finished products and are increasingly looking to *in vitro* methods for this purpose.

Industry has already adopted *in vitro* methods for some other endpoints for the assessment of mixtures and finished products. The methods described in OECD TGs 439 and 492 (skin and eye irritation respectively) have been widely used to assess complex mixtures at a regulatory level for some time, even though the original validation studies only included individual chemicals. So there is a precedent for using the *in vitro* tests for a variety of formulations, but how does this currently apply to the skin sensitisation methods? All three OECD TGs state that the tests can be used with certain restrictions. The DPRA is limited to mixtures of known composition. Ideally, any in-depth validation of the methods for use with mixtures and finished products would need to be sector-specific, using relevant reference mixtures for each type of application. Such an approach would be complex and expensive and requires industry-wide collaboration.

**PROMISING PILOT STUDIES**

There are several interesting studies in the scientific literature, evaluating mixtures and finished products. These include a promising pilot study published by Natura and Givaudan (Andres *et al.*, 2013), demonstrating that the KeratinoSens™ test works for a variety of plant extracts and can detect minor components with sensitising potential, while Settivari *et al.* (2015) showed strong predictivity for a set of 10 agrochemical formulations.

**GHS GUIDELINES**

The GHS Guidelines published by the International Labour Organisation (ILO) also provide some useful information about assessing the sensitisation potential of mixtures using read-across from available data on individual ingredients, and includes “bridging rules” such as allowances for dilution, batch-to-batch variability and additional read-across from similar mixtures that have been more fully characterised. This type of assessment could be usefully supplemented by *in vitro* data on the mixture itself.

To date, there is relatively limited published information available, but in our experience, the *in vitro* skin sensitisation tests are undoubtedly providing useful data that contributes to informed product development as well as building a weight of evidence for safety assessment.
As a fast-growing global cosmetics brand, Lush has always been a leading voice in campaigns against animal testing, and has set up the Lush Prize to reward and fund the development of animal-free alternatives.

Since winning the Lush Prize in 2013, XCellR8 has developed a strong partnership with the company, and as part of our ongoing programme we have tested over 140 finished cosmetic products using the DPRA and KeratinoSens™ tests. The finished products include shower gels, soaps, bath bombs, fragrances and a variety of skincare formulations. We have also tested a wide range of complex mixtures including essential oils, absolutes, resins and natural extracts. The benefits of the ongoing testing programme include:

• Using the results as supporting information for the safety assessment process for finished products and as a prelude to testing on human volunteers.
• Valuable input to new product development. Ongoing testing of finished products supplements the results for individual ingredients and supports the formulation and product development process.
• Interpreting the emerging patterns of data to learn more about the predictive capacity of the tests for complex mixtures and finished products, bringing knowledge to benefit the wider industry.

“Our vision for safety testing at Lush has always been to eliminate animal tests, not just for ethical reasons but because we are unhappy with the scientific validity of historic animal-based methods. Our partnership with XCellR8 enhances the safety of our customers and brings benefits for the whole industry.”

KARL BYGRAVE, DIRECTOR, LUSH
Testing finished cosmetic products for Lush (continued)

XCELLR8’S UNIQUE ANIMAL-PRODUCT-FREE APPROACH:

It’s really important to highlight that the majority of in vitro tests utilise animal-derived components such as serum, tissue extracts and antibodies, so they can’t be considered truly animal-free.

XCellR8’s laboratory is unique in our completely animal-product-free approach. Our partnership with LUSH has enabled us to uniquely adapt the KeratinoSens™ and h-CLAT tests to animal-product-free conditions and to carry out a validation of the adapted methods using the panels of reference chemicals stipulated in the relevant OECD TGs and Performance Standards. The KeratinoSens™ adaptation was published in the ALTEX journal (Belot et al., 2017) and has been approved for inclusion in OECD TG 442D – signifying full regulatory acceptance.

The h-CLAT adaptation was also recently published in ALTEX (Edwards et al., 2018) and is currently in the review process for potential inclusion in TG442E. Meanwhile, it may be used in REACH registrations in combination with the performance data.

DPRA RESULTS FOR A BATH BOMB

Sample skin sensitisation test results for a bath bomb. None of the concentrations caused >1.5-fold induction of the response in the KeratinoSens™ test, and peptide depletion in the DPRA was categorised as "Minimal". The bath bomb was therefore classified as a non-sensitiser using the “2 out of 3” approach. The results were used as supporting information for safety assessment purposes and to contribute to an internal database for ongoing product monitoring and development.

www.x-cellr8.com
Definition of the AOP for skin sensitisation over the past decade has led to the successful development and validation of three in vitro tests, resulting in the publication of OECD TGs 442C (DPRA), 442D (KeratinoSens™) and 442E (h-CLAT).

Current regulatory guidance recommends a “two out of three” approach using these tests to classify sensitisation potential.

The in vitro skin sensitisation tests are now the default requirement for a variety of regulations including REACH, and methods once known as “alternatives” are now mainstream.

The tests may be used to discriminate between sensitisers and non-sensitisers in the context of an IATA. They cannot be used to sub-categorise sensitisers into UN GHS class 1A / 1B or to formally predict potency. However, they can potentially contribute to the assessment of potency as part of an integrated approach.

All three tests may be used for mixtures and finished products, subject to certain compatibility criteria such as solubility. The DPRA is limited to mixtures of known composition. It must be remembered that the methods have not been formally validated for mixtures and there are various limitations, but the data can be very useful as part of a weight of evidence approach and for product development purposes.

None of the current methods incorporate metabolism, so there are limitations when assessing pro-haptens and pre-haptens, although recent research suggests this may not be a major barrier to correct classification.

The current limitations including those around potency and metabolism are likely to be the focus of the next developments in the field, and new methodologies are set to be embraced for this purpose, including the use of reconstructed tissue models and increasingly sophisticated computer-based (in silico) approaches. The adoption of genomic technology into skin sensitisation testing (GARDpotency™) is an exciting development with great potential.

Currently, there is much discussion about the importance of linking back test results to real-life exposure, and a major study by the Cosmetics Europe Skin Tolerance Task Force (Reisinger et al., 2016) stressed the importance of incorporating factors such as bioavailability / absorption and skin metabolism data.

A huge amount of progress has been made in the field of in vitro skin sensitisation testing in the past decade and this is a cause for major celebration, both in terms of the advancement of science to better protect human health and for enhanced ethical and sustainable approaches. We’re very excited to be involved in this journey and to continue to make discoveries in the decades to come!
## At-a-glance jargon buster

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<thead>
<tr>
<th><strong>AOP</strong></th>
<th>Adverse Outcome Pathway</th>
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<tr>
<td><strong>CLP</strong></td>
<td>The CLP Regulation (for “Classification, Labelling and Packaging”) is an EU regulation aligning the European Union system of classification, labelling and packaging of chemical substances and mixtures to the Globally Harmonised System (GHS).</td>
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<tr>
<td><strong>ECHA</strong></td>
<td>European Chemicals Agency. An agency of the EU responsible for the enforcement of REACH and CLP.</td>
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<tr>
<td><strong>IATA</strong></td>
<td>Integrated Approaches to Testing and Assessment: pragmatic, science-based approaches for chemical hazard characterisation that rely on an integrated analysis of existing information coupled with the generation of new information using testing strategies.</td>
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<tr>
<td><strong>LLNA</strong></td>
<td>Local Lymph Node Assay. Animal (mouse) based test for skin sensitisation: OECD TG 429.</td>
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<tr>
<td><strong>Pre-hapten</strong></td>
<td>A chemical that can convert to metabolites without any biological enzyme system.</td>
</tr>
<tr>
<td><strong>Pro-hapten</strong></td>
<td>A chemical that requires conversion by enzymes to exert its sensitisation potential.</td>
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<tr>
<td><strong>REACH</strong></td>
<td>An EU regulation designed to improve the protection of human health and the environment from the risks posed by chemicals. REACH stands for Registration, Evaluation, Authorisation and Restriction of Chemicals.</td>
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<tr>
<td><strong>TG</strong></td>
<td>OECD Test Guideline</td>
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