Inside *in vitro* testing

Cosmetic claim support explained

XCELLR8

Redefining testing

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About the author

Dr Carol Treasure first witnessed animal testing while working in the pharmaceutical industry and during her degree in Physiology and Pharmacology at Sheffield University, and resolved then that there had to be a better way, not just ethically, but scientifically, too.

She went on to complete a PhD at FRAME (Fund for the Replacement of Animals in Medical Experiments), followed by seven years with Cascade Biologics in Portland, Oregon. Setting up Cascade's European operation in 2003, Carol was able to use her scientific knowledge in a commercial context, preferring an environment where her skills could be used directly to make a positive impact on the industry and ultimately, replace animal testing.

When Cascade was acquired by a big corporation, Carol co-founded XCellR8 with Bushra Sim in 2008. The company looks after the testing programmes for high street giants, large ingredient manufacturers and SMEs alike. Its award-winning work has been recognised at a regulatory level by the OECD and the European Chemicals Agency (ECHA), and supported by top funding bodies such as Innovate UK and the European Horizon 2020 programme.

Redefining testing

XCellR8 provides animal-free safety testing and efficacy claim support to the cosmetic and chemical industries.

We help companies to develop safe and innovative new products, to comply with legislation, and to adopt animal-free testing strategies that are both scientifically advanced and ethically sound. We are the only testing lab globally to offer a completely animal-free approach to testing, by eradicating all animal-derived components from our *in vitro* methods.

Our most commonly used efficacy tests include anti-oxidant activity, mildness, skin pigmentation and collagen expression and we frequently develop bespoke solutions for *in vitro* claim support work. Turnaround times can be as short as four weeks.

Get in touch today to discuss your testing requirements.

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Introduction to *in vitro* technologies

In vitro technologies have the power to provide real claim support data and optimise your product development process. With regulatory changes driving the expansion of *in vitro* methods, a wide number of efficacy and safety tests are now catered for. Together we'll explore some of the latest developments of *in vitro* technology, assess their potential and share how you can ensure robust data is produced.

Much to celebrate!

But what about the applicability of *in vitro* methods for supporting safety and efficacy claims? Well, there's much to celebrate! The past decade has seen an explosion of new technologies in this field and *in vitro* methods are providing extremely valuable claim support data at an early stage of development, applicable to both novel ingredients and final formulations. Key efficacy claims supported by *in vitro* data include mildness, skin lightening, self-tanning, anti-blemish, anti-ageing and anti-inflammatory. A great new test for anti-oxidant activity in skin cells is available to support anti-pollution claims. In many cases, the tests can provide key pieces of information prior to finalising a formulation for testing on human volunteer panels.

Increasingly, *in vitro* data is also being used to provide supporting information for safety claims. Some of the regulatory tests can be adapted for this purpose. For example, the new regulatory skin sensitisation tests can contribute useful data to claims that products are hypoallergenic and, in combination with skin irritation tests, can assess whether a formulation is suitable for use on sensitive skin. Interestingly, *in vitro* methods may also have a place in helping to address public concerns over the safety of certain highly publicised ingredients such as parabens, providing a fast, direct and robust way to generate human-relevant data.

An important note of caution

Whether you are thinking of using *in vitro* data to support either safety or efficacy claims, these tests provide fantastic supporting information but do not necessarily act as "stand-alone" evidence for claim substantiation, even if they have regulatory approval. In the absence of a definitive regulatory framework for the application of *in vitro* data in the field of claim support, each ingredient or formulation must be carefully considered on a case-by-case basis. The results must always be applied in compliance with local regulations and advertising standards in the relevant territories of the world where you intend to sell the product. Given the rapid increase in the availability of reliable *in vitro* tests for claim support purposes, there is a clear need for such a framework as a guide for product developers, as there is no doubt that these sensitive tests provide valuable, mechanistic information that is often more detailed and conclusive than human trials alone.

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Test Systems

Human cell cultures

Let's take a look at some examples of useful *in vitro* models. The simplest approach – and one that is more than adequate in many cases – involves the culture of a single layer of human skin-derived cells. Such methods provide a fast and cost-effective screen, but certain practical limitations mean that appropriate compatibility checks should always precede the study. These include checking the solubility of the ingredient or formulation in aqueous cell culture medium (sometimes this requires pre-dissolving in solvents such as DMSO or ethanol) and a basic cytotoxicity screen to determine the concentrations that the cell cultures will tolerate during the main test. It's important that cells are sourced from approved sources, with full donor consent. Most commonly, the cells are derived from waste tissue following plastic surgery procedures including breast reduction or abdominoplasty ("tummy tuck"), and are available from single or pooled donors. Cells should always be screened for the absence of viruses such as HIV and hepatitis, as well as mycoplasma – which not only present a hazard to worker safety but subtly impact the cells' responses and behaviour. They should also have been fully QC checked prior to use, for aspects such as morphology in culture, growth rates, and sterility. Epidermal keratinocytes and melanocytes, and dermal fibroblasts, are all available to such standards.

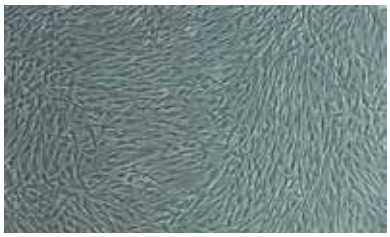

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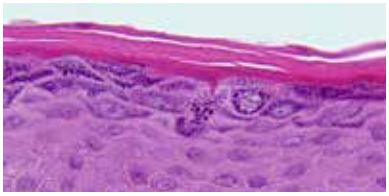
Figure 1: Human skin cells. Keratinocytes from the epidermis (a) are used to measure anti-oxidant activity, while fibroblasts from the dermis (b) show whether an ingredient or formulation has collagen-boosting properties. At XCellR8, all cells are cultured in an animal-product-free environment, providing the closest possible simulation of human physiology and avoiding ethical issues around the use of animal-derived test components.

At XCellR8, all cells are human-derived and we also eradicate any animal-derived components from the culture system, replacing them with human sources. The culture of human cells in an animal product-free environment not only ensures maximum physiological relevance, but also provides the ethical advantage of avoiding the need to sacrifice animals to support cell culture work. Traditionally, many "*in vitro*" methods have utilised animal-derived products such as serum and liver extract – a practice that is often overlooked and requires the sacrifice of animals. This is no longer necessary, with the ready commercial availability of human-derived equivalent products.



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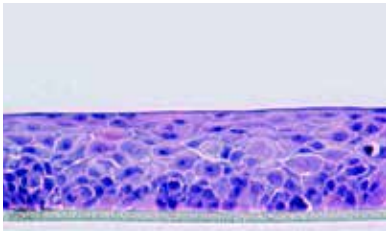

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Figure 2: Human reconstructed tissue models. Tissue engineering technology is used to recreate human skin (a) and cornea (b) *in vitro*, enabling a variety of elegant test methods, including assessment of irritation potential or mildness. Cosmetic ingredients or final formulations are dosed directly to the tissue surface, closely simulating real-life exposure. (Images courtesy of Mattek Corporation).

Reconstructed ("3D") human tissue models

The availability of reconstructed human skin and eye tissue has enabled the development of some elegant and sophisticated tests for cosmetic safety and efficacy. These accurate 3D models are based on tissue engineering technology, using human cells to recreate close simulations of human tissue under carefully controlled conditions. Importantly, reconstructed human skin models possess a *stratum corneum* and therefore have a barrier function. While the barrier tends to be more permeable than that found in healthy human skin, it does represent a significant advancement in providing physiologically relevant models for use in testing. 3D models also circumvent the need for test items to dissolve in aqueous cell culture medium, and allow ingredients or formulations to be added directly to the skin surface, mimicking real life exposure. Models are available in a number of specialised formats, including epidermis only, and full-thickness skin (with a dermal fibroblast layer, often embedded in a collagen gel).

Claim Support Tests

Anti-oxidant claims

Anti-oxidant activity has become a very popular phrase, and is relatively well-understood by consumers due to its overlap with food and nutrition. Since reactive oxygen species are produced in the skin in response to a wide range of factors including stress, pollution and the natural ageing process, the claim has wide-ranging applications. Human epidermal keratinocyte cultures are an accessible and effective model for assessing potential anti-oxidant activity in a wide range of test items, from novel active ingredients and extracts through to final formulations. Unlike anti-oxidant tests that utilise simple biochemical reactions in a test tube, the use of whole cells provides a more complete environment with higher physiological relevance, accounting for important aspects such as pH, cellular uptake and metabolism, as well as efficacy of the test item.

At XCellR8, we have validated a test method called OxiSelect™ using human keratinocytes in animal-product-free culture. The cells are exposed to a range of concentrations of the test item. A marker compound is then taken up by the cells and rapidly oxidised to a fluorescent product by reactive oxygen species (ROS) in the presence of a free radical initiator molecule. In the presence of an anti-oxidant ingredient or formulation, the reaction is quenched and a lower fluorescent signal is observed. The plant flavonoid, quercetin, is used as a positive control. Another big advantage of this test is its ability to capture anti-oxidant contributions from all major types of reactive oxygen species, including peroxides, superoxides, hydroxyl radicals and singlet oxygen.

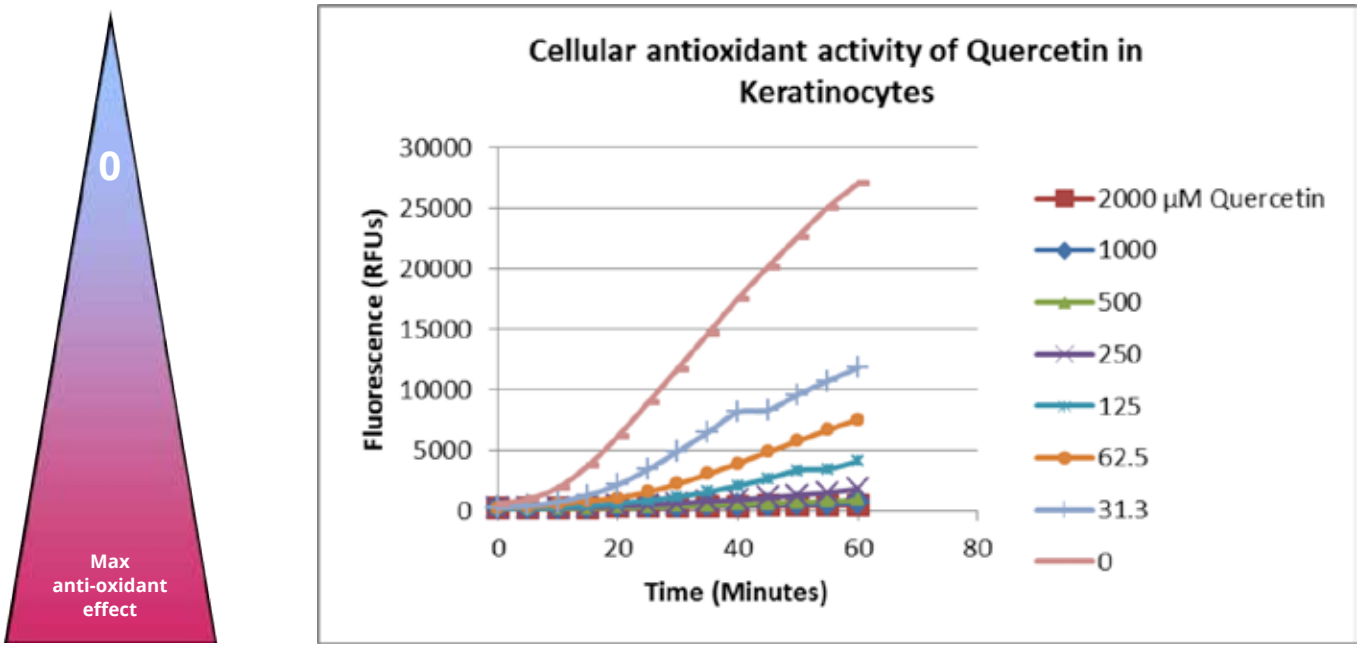


Figure 3: The OxiSelect™ *in vitro* test for anti-oxidant activity. Human skin cells (epidermal keratinocytes) are grown in animal-product-free culture conditions and exposed to a range of concentrations of the active ingredient or formulation. Anti-oxidant activity inhibits the production of the fluorescent signal by the cells. Graph demonstrates the dose-dependent effect of the plant flavonoid, quercetin, used as a positive control in the test.

Mildness claims

Reconstructed human skin and eye models provide a very effective system for assessing the mildness of ingredients or formulations. Using an adaptation of a regulatory skin irritation test, subtle differences between mild and ultra-mild formulations can be measured, allowing a series of products to be placed into a rank order of irritation potential (and therefore mildness).

The method enables assessment of unknown formulations against industry benchmarks. For example, the skin models can be used to assess the mildness of novel surfactants against classic surfactants – a need that has been highlighted by cosmetic companies who find some of the traditional tests (such as HET-CAM and RBC) to be "inadequate and out of date", lacking relevance and the required level of sensitivity. The eye models are commonly used to assess new formulations of baby shampoos, for example, to compare to standard benchmark products. The methods can also be used to provide supporting information for safety claims such as "suitable for sensitive skin/eyes", "suitable for all skin types" and "no tears." They provide a quick and cost-effective pre-screen, prior to human volunteer studies, providing a range of commercial and ethical benefits and enabling selection of the mildest formulations at an early stage of product development.

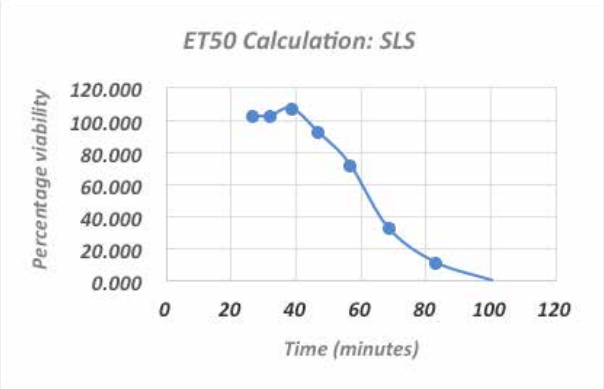


Figure 4: Mildness testing using reconstructed human tissue models. Ingredients or formulations are applied to the tissue surface, and damage to the cells is measured over time. The "ET50" value (time taken to reduce the viability of the cells) is calculated relative to untreated controls and can be used to calculate a rank order of irritation potential, enabling a powerful mildness prediction for a series of ingredients or formulations relative to each other or to industry benchmarks. A prediction model also extrapolates the ET50 to an irritancy classification (Severe, Moderate, Mild or Minimal / Non-Irritating).

Mildness tests are used to assess baby product formulations

Sensitive skin

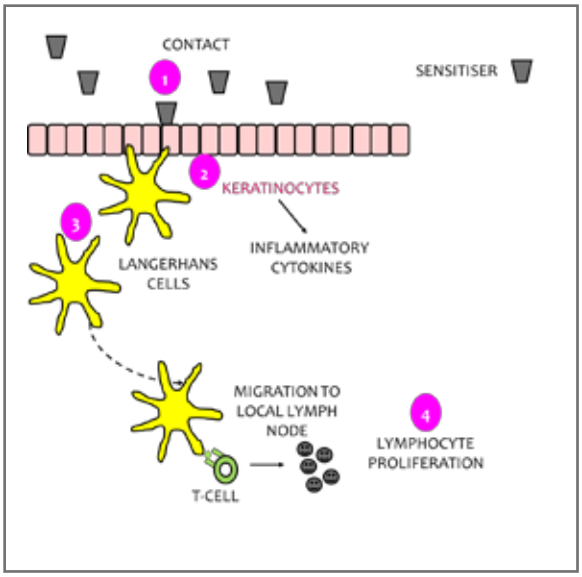
The term "sensitive skin" is widely used and can refer to skin irritation and / or sensitisation. Skin irritation involves short-term, reversible damage to the cells of the epidermis (keratinocytes). It is a one-off event and can be effectively measured using the same "ET50" method as described earlier for mildness.

Skin sensitisation, on the other hand, is a much more complex process involving an immune response. Once a consumer is sensitised to a particular cosmetic ingredient, the condition is, in theory, lifelong, so avoiding sensitisation potential is a crucial element of any cosmetic product development process.

Three *in vitro* regulatory safety tests are now available for sensitisation, and these can all be used to provide supporting data for safety claims including "suitable for sensitive skin", "hypoallergenic", and "suitable for all skin types".

A powerful prediction of skin sensitisation potential

Again the *in vitro* methods are hugely valuable in the product development process, enabling the formulator to select the ingredients with the best profile for both product safety and claim support purposes. The three available tests model the key events in the skin sensitisation process (protein binding, skin cell activation and immune cell activation). Depending on the application, some or all tests may be used together for a powerful prediction of skin sensitisation potential. While standard versions of these tests use animal-derived components, XCellR8 has uniquely created and validated animal-product-free adaptations in response to requests from cosmetic companies and gained approval for the adapted methods at OECD level.



KEY EVENTS IN SKIN SENSITISATION AND RELATED TESTS:

1) FIRST CONTACT & BINDING TO SKIN PROTEINS :
Direct Peptide Reactivity Assay – DPRA : OECD Test Guideline 442c

2) RELEASE OF PRO-INFLAMMATORY CYTOKINES BY KERATINOCYTES :
KERATINOSENS™ : OECD Test Guideline 442d

3) DENDRITIC CELL ACTIVATION/ MATURATION :
Human Cell Line Activation Test – h-CLAT: OECD Test Guideline 442e

4) T-CELL PROLIFERATION – no validated *in vitro* test currently available.

Figure 5: The skin sensitisation pathway. *In vitro* tests are available to model key events 1-3, and may be used in a supporting role when developing products for sensitive skin.

Skin pigmentation

The close interaction between keratinocytes and melanocytes is known to be a critical factor in regulating melanin production and pigmentation. A particularly exciting development involves the incorporation of melanocytes into reconstructed human skin models. In one such model (MelanoDerm™), human melanocytes are incorporated into the epidermis in an approximate ratio of 1 melanocyte to 10 keratinocytes, where they locate to the basal layer, closely simulating real-life conditions.

Variations of the model are available using melanocytes derived from donors of different ethnic origins, increasing the direct relevance to target groups for specific products. The model can be used to assess the efficacy of skin lightening formulations, self-tanning products and dark spot correctors. Following exposure to the test item, pigmentation is recorded using photographic techniques to provide a qualitative assessment, followed by extraction of melanin from the skin models and quantitative analysis by spectrophotometry. The MelanoDerm™ models are viable for up to two weeks in culture, providing the potential to extend test protocols where relevant for longer-acting ingredients and products.

'Reconstructed skin models can now simulate conditions that regulate melanin production'

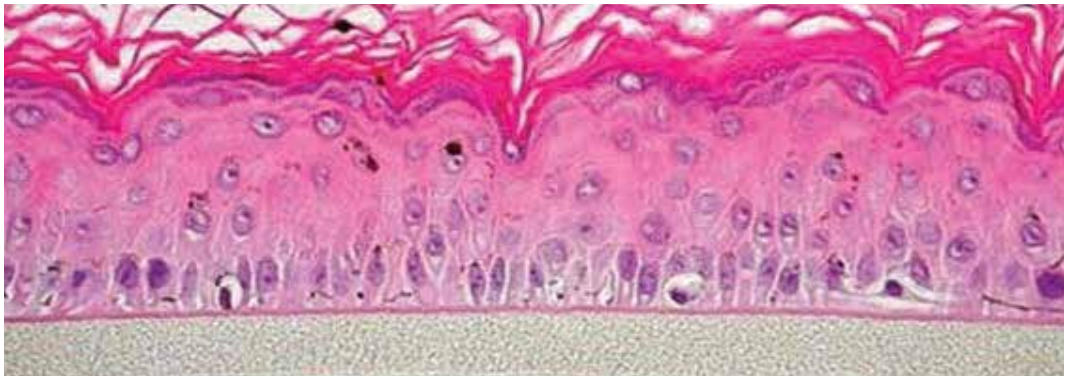


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Figure 6: The MelanoDerm™ skin model (a), incorporating human melanin-producing cells (melanocytes) for the efficacy assessment of ingredients or formulations to modify pigmentation, such as dark spot correctors. Items are applied to the surface of the skin model (b) and incubated for specified time points. The impact on pigmentation is assessed photographically and quantified by spectrophotometry. (Images courtesy of Mattek Corporation).

Anti-ageing

Human dermal fibroblast cultures are increasingly used as a preliminary screen for key anti-ageing effects such as stimulation of collagen I synthesis and inhibition of MMP-1 (matrix metalloproteinase 1 – an enzyme involved in collagen breakdown during the ageing process).

The most commonly used methodology involves a technique known as Enzyme Linked Immunosorbent Assay (ELISA), which employs labelled antibodies to detect levels of collagen or MMP-1 in the cell cultures. ELISA-based tests are available for a wide range of markers associated with ageing and inflammation. While fluorescent microscopy techniques can also be used to detect the expression of collagen and other markers, quantifying the results is often subjective and less reliable than a quantitative ELISA based test.

ELISA-based tests are available for a wide range of markers associated with ageing and inflammation.

Best practice

When conducting *in vitro studies*, robust experimental design and data analysis as well as appropriate interpretation of the data are critical. While *in vitro* technologies provide an exciting tool box to generate data for claim support, the limitations of each method must also be recognised. Unfortunately, the exaggeration of claims in product brochures is relatively commonplace, a practice that may sometimes only be spotted by highly trained and experienced *in vitro* scientists. Common pitfalls to look out for include:

- Poor choice of cell type and endpoint
- Irrelevant positive and negative controls (or absence of controls altogether!)
- Insufficient replicates to provide a statistically significant result
- Lack of error bars / standard deviations in graphs and data tables
- Manipulation of digital images (such as those of fluorescent markers in cells) to artificially enhance differences between controls and test items

When considering the use of *in vitro* technologies to support your product development, I can't stress enough the importance of thoroughly assessing your requirements with an experienced *in vitro* scientist at the outset. Whether this is done through in-house colleagues or outsourced to a contract laboratory, scoping out the project and recognising both benefits and limitations of the methods is essential, to avoid wasting critical time and resources. Our six key recommendations to help ensure that best practices are observed are listed on the right.

Our six recommendations

1. Work with an expert to ensure robust methodology and experimental design
2. Ensure realistic data interpretation – both for studies you commission, and when reading ingredient brochures
3. Take time to plan, avoiding the significant commercial implications of potentially incorporating "active" ingredients into your formulations based on flawed data
4. Cost cutting can be costly! Ensure that adequate replicates are tested to provide a statistically significant result
5. *In vitro* and human volunteer studies can be a powerful combination, predicting human effects and their mechanisms for the purpose of claim support
6. Use an appropriate combination of in-house and outsourced expertise. (Where needed, consider outsourcing an independent analysis of the data included in ingredient brochures, prior to finalising critical commercial decisions)

Summary

To sum up, *in vitro* methods already provide an extremely exciting tool box to the cosmetics industry, and the palette of available tests is rapidly evolving. *In vitro* approaches can be used as a powerful source of data for claim support purposes, and a wide variety of standard and bespoke methods are available. They can act as a very useful pre-screen, prior to human volunteer studies, and can be used in combination with human data to support your claims. However, the implementation of certain "best practice" principles is critical, in order to generate and interpret data in a reliable way, to avoid "paying later" in the commercial process.

In addition to exploiting the many benefits of *in vitro* methods, it's important to recognise limitations such as solubility issues, physiological differences and the difficulty (currently) in modelling long-term exposure. Provided that best practice principles are observed, the rapid and constant evolution of *in vitro* technologies will continue to provide a world of exciting possibilities for supporting your product claims. Watch this space!

To ask for further technical information or case studies on the tests mentioned in this guide, get in touch with XCellR8 today.

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