How do you define truly animal-free testing?

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Abstract

Testing of cosmetics and their ingredients on animals has been banned for many years, yet *in vitro* testing still makes widespread use of animal products and derivatives. Some of these components are harvested 'humanely' or are the by-products of the meat industry. However, some, such as foetal bovine serum, are obtained only via inhumane practices. As well as being detrimental to animals, these additives can reduce the reproducibility of tests. Here we provide some guidance on how to define truly animal-free *in vitro* testing, and describe some of the principles that we follow. We also introduce our scale for animal-free testing, which shows how 'animal-free' methods are. The scale will help stakeholders communicate how 'ethical' their testing is and can be.

Keywords

- *In vitro* testing
- Animal-free
- Animal-product-free
- Animal components
- Cruelty-free
- Cell culture
- Testing

IS IT TRUE THAT COSMETIC PRODUCTS SOLD IN THE EU ARE NOW TESTED WITHOUT CRUELTY TO ANIMALS?

There has been huge change over the last three decades in the testing of cosmetics and personal care products, driven by consumer lobbying across the EU and by regulators. 1993 saw the introduction of a ban on the testing of cosmetic products on animals with the 6th Amendment to the EU Cosmetics Directive. After several delays in implementation of this ban, the 7th Amendment was passed in 2003, stipulating a phased-in ban on animal testing for cosmetic products and their ingredients sold in the EU. This ban became fully operational in 2013 – 20 years after public opinion had successfully driven the legislation for the original ban.

Consumers are increasingly holding cosmetic brands to account; they don't want products to be tested on animals, but they do still expect them to be safe for them and their families to use. Recent data shows that one-fifth of consumers are looking for 'not tested on animals, cruelty-free, and/or 100% vegan' when choosing colour cosmetics or skin care products (1). So, can we now say with confidence that cosmetic products sold in the EU are tested without cruelty to animals? Sadly, no.

IN VITRO DOES NOT NECESSARILY MEAN FREE FROM ANIMAL COMPONENTS

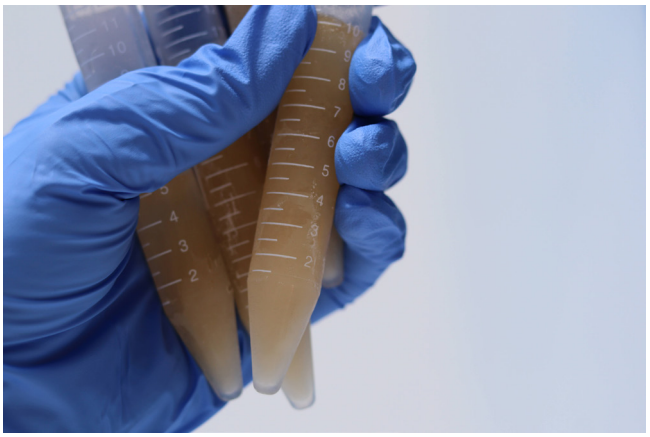
Although only *in vitro* (or *in silico*) safety tests may be conducted on cosmetic products and ingredients, the term *in vitro* does not necessarily mean animal-product-free. Many *in vitro* tests rely on animal components. Most cell culture, even using human-derived cell lines, relies on foetal bovine serum (FBS) for the maintenance of the cells. FBS is derived from blood extracted by cardiac puncture of living, unanaesthetised, calf foetuses (2). Antibodies used widely in many *in vitro* tests are derived from laboratory animals bred solely for antibody production, while *in vitro* metabolism studies commonly rely on rat liver extracts to metabolise chemicals. In some instances, mice are given cancers to allow for the production of supplements for cell culture. Other tests, such as the bovine corneal opacity and permeability (BCOP) test (3), use by-products of the meat industry.

By most peoples' standards, tests using these materials cannot be described as animal-free, but they can be termed *in vitro*, and they do comply with the EU cosmetics test ban.

IS IT REALISTIC TO THINK WE CAN REPLACE ALL ANIMAL COMPONENTS?

Is it even possible to replace all the animal components used in *in vitro* tests with animal-product-free alternatives? In many cases, yes, it is. There are animal-product-free versions of many of the animal-derived components currently used in *in vitro* tests:

- Antibodies can be created using recombinant gene technology instead of using animals
- FBS can be replaced with ethically derived human serum or, better still, the use of chemically defined media
- Rat liver extracts can be substituted with ethically sourced human liver extracts



Ethically derived human serum

However, there are historical barriers to the use of animal-product-free protocols in regulatory approved *in vitro* tests. Since FBS is such a ubiquitous component of cell culture media, most *in vitro* regulatory tests specify the use of FBS in the method, even if it is of questionable scientific and ethical value. The 'habit' of FBS use is continued without considering the consequences of the status quo, or the availability and benefits of new improved approaches.

ANIMAL-PRODUCT-FREE CAN BE JUST AS EFFECTIVE

We have demonstrated that the OECD skin sensitisation tests TG 442d KeratinoSens™(4) and TG 442e h-CLAT (5), which were originally approved for FBS-containing culture media, are in fact just as effective when conducted using animal-product-free media. We have submitted the validation of these amended tests for OECD approval. The OECD have already approved our modified KeratinoSens™ test (in 2018), and approval is pending for our h-CLAT assay, with further work and discussion ongoing. Unfortunately, many *in vitro* regulatory tests do not yet have approved, wholly animal-free, alternatives, but they do at least offer *in vitro* alternatives to live animal tests.

THE CHALLENGE OF DEFINING TRULY ANIMAL-FREE

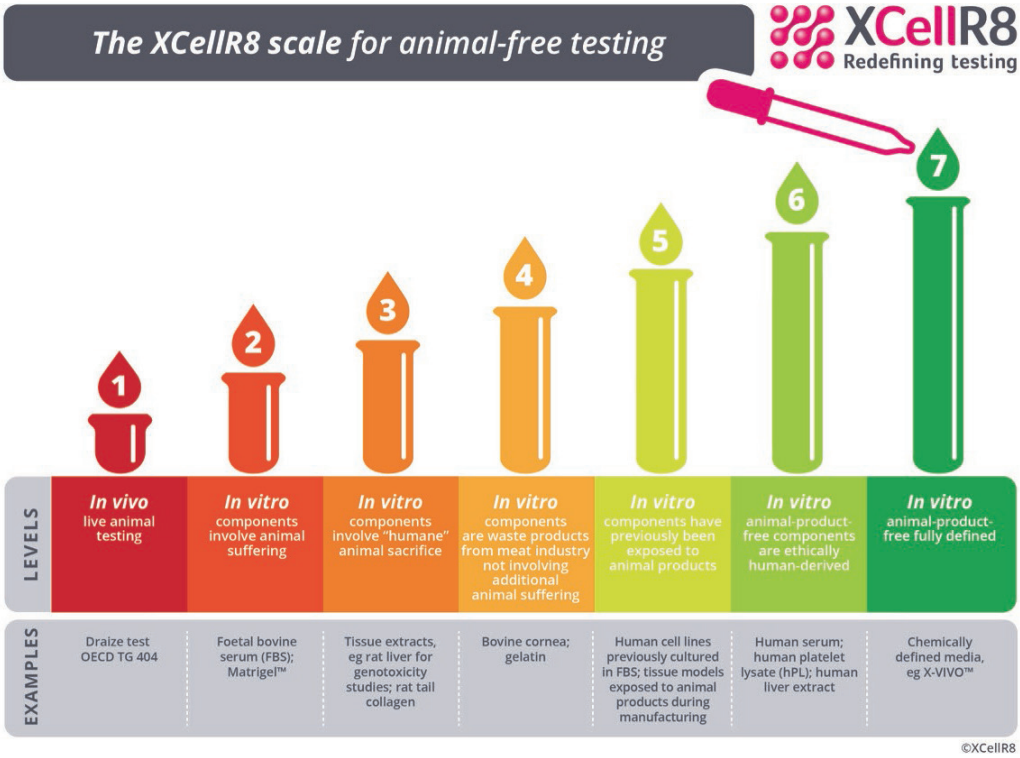
So how do you define truly animal-free testing? Even this is complicated. Almost every transformed cell line grown in *in vitro* culture started its history being cultured in medium containing FBS. It is a commonly held view among scientists that serum is a necessary component of medium to establish and maintain new cell lines. Supposing a cell line is converted to chemically defined media, does the fact that it originated in FBS-containing medium stop it from being considered as animal-product-free? Also, most antibodies used in safety assays are monoclonal and are produced by cultured cell lines. However, these monoclonal cell lines are initially generated using the immune response of live animals, so are probably not fully animal-free. It's worth noting that Nobel Prize-winning 'phage display' technology allows the production of recombinant antibodies without the involvement of live animals.

Unfortunately, there is a long tradition of continuing to use animal-derived material that was available in the early days of *in vitro* biomedical research to support cell culture, even though modern science and technology has brought us more scientifically valid alternatives.

THE XCELLR8 SCALE FOR ANIMAL-FREE TESTING

Our mission is to accelerate the world's transition to 100% animal-free testing. To help guide us in achieving our mission we have created an ethical scale that we use to inform our decision-making.

Categorising the use of animal components like this helps us remember that the vast majority of *in vitro* tests using cell culture are only at Level 2 on our scale, since they use FBS in the culture media. Even though Levels 2-7 are all described as *in vitro*, there are big differences between them, so not all *in vitro* methods are equal when it comes to their animal-product-free status.



What's more, a high proportion of cross-sector chemical safety testing in the world today is still carried out on animals. Even in the UK, where the majority of skin and eye irritation / corrosion tests are conducted *in vitro*, there were still over 100 Draize tests on live rabbits in 2018 (6).

Clearly, and despite the EU Cosmetics testing ban, most testing today is carried out only at Level 1 or Level 2. We are committed to accelerating a move away from product testing at these levels, and to see more scientifically and ethically advanced animal-product-free approaches become standard practice.

For us, animal-product-free testing means following several principles to maximise both the scientific and ethical benefits for our work:

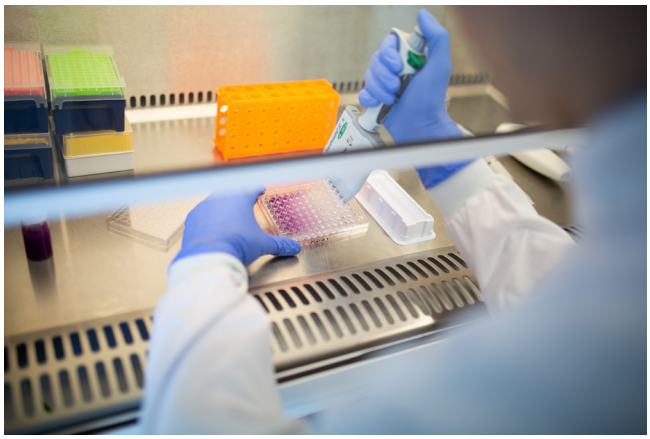
1. Cell lines should be adapted to grow in media that does not contain animal serum. Ideally media should be wholly chemically-defined but, if that is not possible, then ethically-obtained human serum should be used.
2. Only human-derived cell lines, rather than animal cell lines, should be used. From a scientific perspective, for testing the toxicity of chemicals to humans, the use of human-derived cell lines is essential.
3. Protein-based components (e.g. antibodies, extracellular matrix proteins, media supplements etc) should all be derived from recombinant or human sources wherever possible.
4. Tissue extracts (eg liver extracts used to incorporate metabolic capacity into genotoxicity tests) should all be from human sources.
5. The generation of test components, such as reconstructed human tissues, should avoid the routine use of animal-derived elements in the production process wherever possible.
6. Our supply chain needs to be both monitored to ensure that we source animal-product-free materials when we set up new tests, and regularly audited to maintain compliance.
7. Tests should always be conducted in a way that achieves the highest possible level of animal-product-free compliance (Levels 1-7 as described in the scale).

These principles guide us in our journey to offering *in vitro* tests that provide the most human-relevant science, and the most practical animal-free ethical position, as we try to fulfil our mission.

WHAT IS THE ANSWER WHERE ANIMAL-FREE TESTS DON'T EXIST?

What if completely animal-free testing is not possible in some instances? This is a challenging dilemma; certain tests must be conducted in order to meet safety regulations, and yet there are not always wholly animal-free methods available to perform these tests.

One example of this deficit is the traditional acute toxicity 'six-pack' of tests which still requires animal tests for some categories. The six pack comprises tests for acute toxicity via oral, skin, and inhalation exposure, as well as through eye and skin irritation and skin sensitivity. The OECD test guidelines have been updated to apply 3Rs principles to the acute toxicity tests, thereby reducing the number of animals used, and methods have been improved to reduce the use of lethality as an end point. However, there is as yet no validated *in vitro* test for acute toxicity.



We have developed a non-regulatory screen for oral acute toxicity using human cells in animal-product-free culture, and have carried out a preliminary validation using 20 cosmetic ingredients. This method is being used by companies to help build a weight-of-evidence for regulatory submissions and to avoid animal testing. We are committed to building further on this research as well as aligning with wider industry initiatives to develop animal-free approaches in this area (7).

In the case of the skin- and eye- specific tests, there are approved *in vitro* alternatives that avoid the use of animals in many cases, but these *in vitro* alternatives may still use animal components (like the BCOP test mentioned earlier).

One approach that we take is to explore certain areas where we believe that we can develop new, animal-product-free tests. With the support of UK and international funding bodies, and industry partners, we are able to ensure continued progress.

TRANSPARENT COMMUNICATION IS VITAL

It's clear that *in vitro* doesn't equate to animal-free, and that *in vitro* tests can, and often do, use animal-derived material. We feel that transparency and communication are the key to building trust between Contract Research Organisations and the personal care and the wider chemical industries. If current technology does not enable a test to be conducted in a wholly animal-free way, then clearly stating this reality is essential so that companies are fully aware of the extent to which their *in vitro* testing programme is animal-free. The scale for animal-free testing is a valuable tool to support these discussions.

It is critical that all of us in the consumer-facing industries communicate the current reality of *in vitro* testing to consumers in the high street and in online stores. Vegan or cruelty-free marketing positions must be founded on truth and clarity. Manufacturers of finished products should be

able to provide an explanation, and evidence, of how their products and ingredients have been tested. Beyond explaining the current limitations of *in vitro* testing technology, it is also essential that we, both at XCellR8 and collectively as an industry, work hard to develop a pathway that leads to more scientifically relevant product testing that can truly be described as animal-free.

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