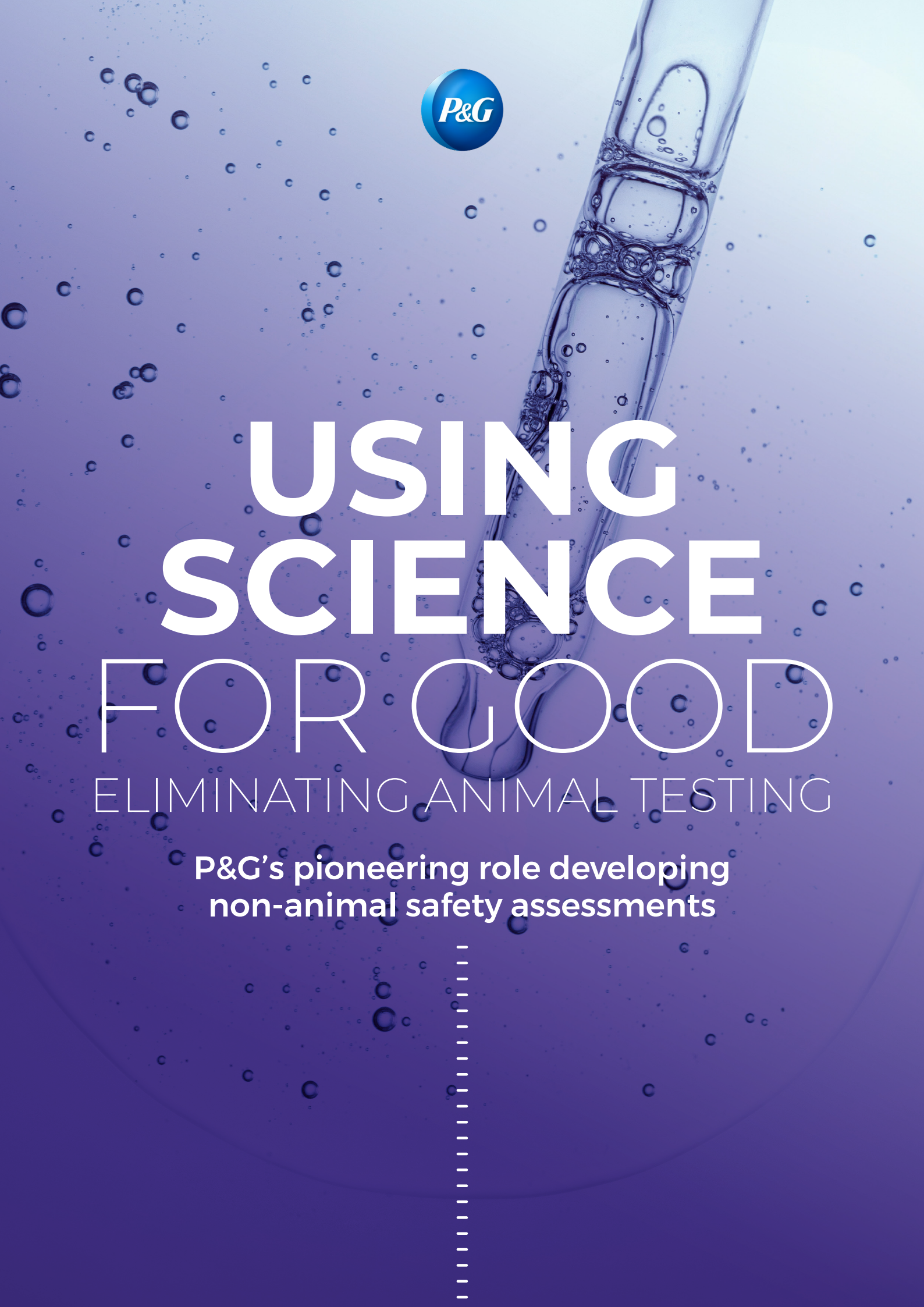


USING SCIENCE FOR GOOD

ELIMINATING ANIMAL TESTING

P&G's pioneering role developing non-animal safety assessments

Introduction

At Procter & Gamble (P&G), we are passionate about safety. Our commitment to delivering high-quality, safe products has been central to everything we have done over the past 180 years. This is why we currently have a team of over 500 scientists engaged in verifying the safety of all our products, from personal care products to baby products, and laundry detergent to household cleaners.

Our safety process follows the central science-based principles of leading academic experts and authorities around the world, such as the U.S. Food and Drug Administration, the E.U. Commission's scientific agencies and committees, the World Health Organization, and the U.S. Environmental Protection Agency. Many ingredients we have investigated over the years have never even made it into our products, as they simply did not meet our high safety standards. At P&G, we will never compromise on safety or quality.

Equally important to us is animal welfare. From the very beginning, P&G knew that ccommitting to animal-free testing would be a significant and challenging undertaking that would take years. Undeterred, we have used our passion to achieve the right thing: an ethical safety approach, combining better safety science that is more accurate than ever before. Several decades of effort and innovation have led us to establish the safety of cosmetic products without the use of animals.

Close collaboration with a number of different stakeholders made our work possible. To advance safety science, we have partnered with leading international animal welfare organisations, academia, industry associations, non-governmental organisations, health and safety authorities, and policy makers. This has led to many of our animal-free testing methods being accepted by global health and safety authorities.

P&G Partnerships & Collaborations

We began our animal cruelty-free journey over 40 years ago, working on developing the science, validating methods and approaches, and advocating for their regulatory acceptance. Along with understanding the magnitude of responsibility we hold, we are proud to be playing an important role in driving forward scientific solutions. All of our research is widely available, ensuring that we contribute to the ultimate objective of building capabilities in non-animal alternatives worldwide.

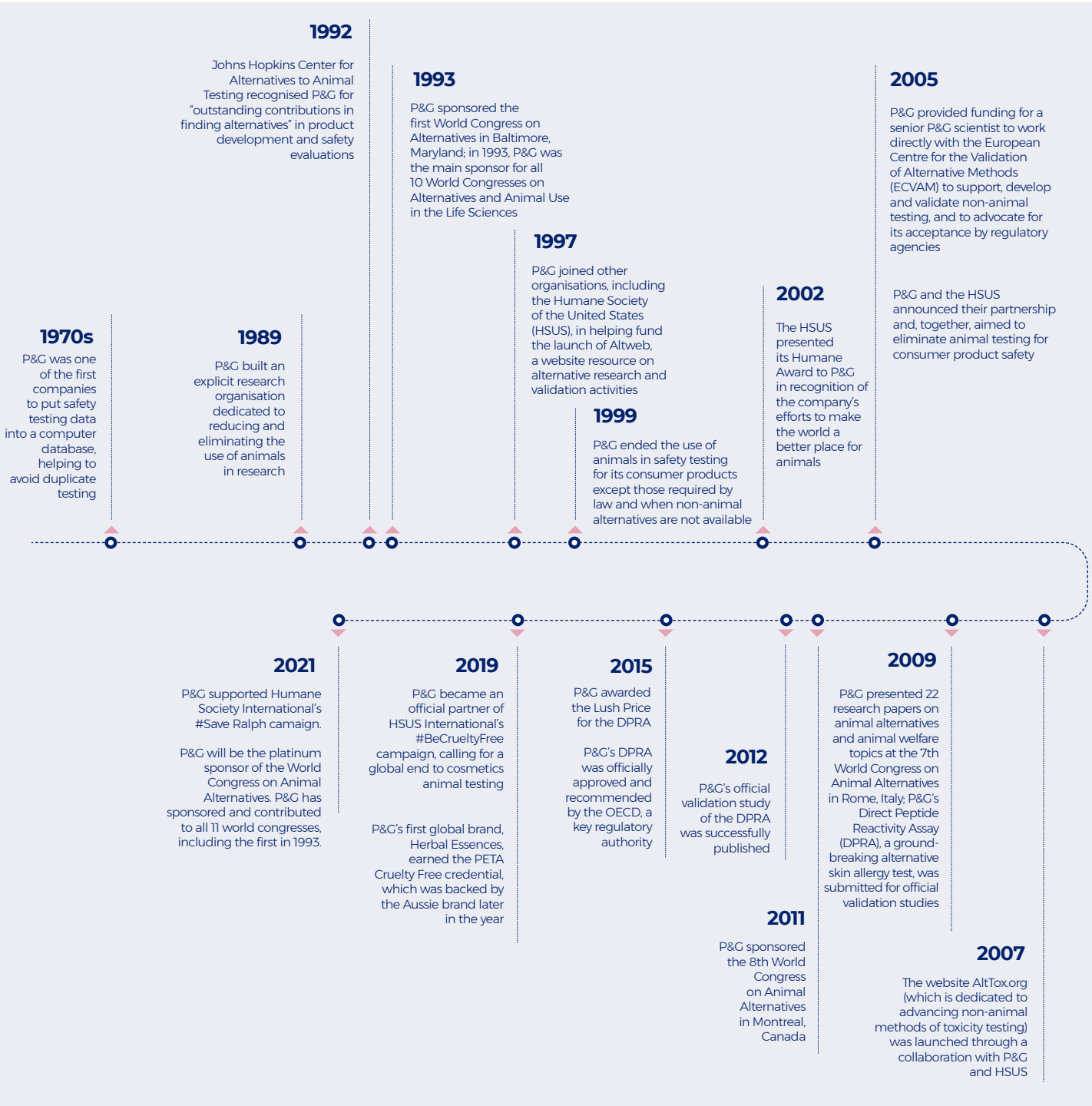


Figure 1. *Timeline of P&G activities in alternatives to animal testing over the past several decades.*

Over the past several decades, P&G has invested over $460 million specifically to **refine, reduce,** and **replace** animal testing – the **3R principle** *(figure 1)*. As a result of our efforts into innovation and continued research, we have developed many effective alternatives to animal testing. To share our message across the globe, we have published our research in over 1000 peer-reviewed research papers in many scientific journals, and presented our work in innumerable international conferences. From its first inception, P&G has been the main sponsor of the World Congress of Alternatives and Animal Use in the Life Sciences.

Our efforts, along with those of our collaboration partners, have allowed us to establish the safety of our personal care products without the need for new animal data. As a result, an ever-growing number of P&G global brands are officially certified by PETA as cruelty-free.

Despite all our efforts, common misconceptions often remain that a product not tested on animals could be less safe or not tested at all. This misconception is unfounded, especially when it comes to our products. At P&G, all our products assessed through non-animal alternative testing methods are established as safe for use by the most rigorous and thorough processes.

Historical safety testing

In the past, animal tests on products and their ingredients were a routine part of safety testing by companies, academia, and health authorities. These tests were designed to assess how an animal would respond to specific ingredients and products. They yielded information and insights on a variety of stages, such as how the product is absorbed and metabolised in the body, whether any organs are negatively affected, and how long the substance remains in the animal's body before it is eliminated.

At P&G, to move away from testing on animals, we needed to devise methods to assess and predict the effect of a product or ingredient on each of these stages through other, non-animal means. To enable this, we first needed to understand the fundamental reactions that take place in the body for each of these effects to occur. Then, we needed to build approaches, and/or prediction models, to enable an evaluation of products and ingredients without having to resort to the use of animals.

Developing non-animal testing methods is a complex, intricate process that requires significant tenacity, scientific acumen, problem-solving, investment, and close collaboration with experts. For decades, P&G has worked with the **3R principle**, refining, reducing, and replacing animal testing.

Many personal care products are designed to be applied to the skin. Some ingredients within products are intended to penetrate the skin, others may penetrate and cause unwanted reactions inside the body. Thorough testing and safety assessments are crucial to minimise any risk to consumers and to confirm a product's safety. The biological understanding of how an ingredient may act in the body and trigger potential adverse reactions has been key to mimic these effects in non-animal alternative methods. This understanding combined with research into different assays, has led to a new generation of safety assessments.

These alternatives not only have an important ethical advantage, but are often more specific and time-effective, enabling researchers to test the safety of ingredients in a controlled environment. In this paper, we share two examples of alternatives to animal testing that have been pioneered by P&G.

Our first example will address **skin sensitisation** or skin allergy and how, from an in-depth understanding of this process, we can assess the potential for adverse effects ingredients in our consumer products when applied to the skin, without testing on animals.

Secondly, we will present **read-across**, our non-animal method to assess the hazard of an ingredient for which toxicity data are lacking and which cannot be generated. This method, using data from related ingredients, aims to predict the effect of ingredients on the skin surface and inside the body, without the need to rely on data on the ingredient itself.

These approaches spare animals from testing experiments worldwide. We are proud to have shared these methods with academia and regulatory authorities for their own direct use.

Alternatives to animal testing for skin sensitisation

WHAT IS SKIN SENSITISATION?

Evaluating potential skin sensitisation effects is pivotal for safety assessments, with primary focus on products and ingredients that are applied to, or come into contact with, the skin. Many ingredients, including those naturally occuring, can trigger an allergic response in susceptible individuals. Substances that can cause an allergic response following skin contact are known as 'skin sensitisers', and may include certain fragrance materials, dyes, preservatives, or plant extracts (such as poison ivy) (1). Importantly, sensitisation is not only a local effect, but involves the systemic immune system (1). However, the allergic response first manifests on the skin, shown as reddened, swollen, itchy patches.

Susceptible individuals who develop an allergy to skin product ingredients will usually remain sensitised for life (1). Skin allergies are not only distressing but also potentially harmful, possibly leading to severe reactions. Ensuring that personal care products and ingredients do not induce skin sensitisation is a key responsibility for manufacturers.

Skin sensitisation can lead to allergic contact dermatitis (ACD), which is an immune-mediated disease. This is triggered by contact of ingredients applied to the skin and is different from 'respiratory' sensitisation, which is usually caused by inhaled or consumed substances (1), and has a different immune mechanism and clinical manifestation.

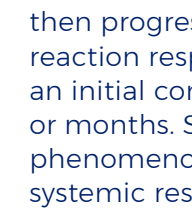
ACD is a common occupational and environmental health problem (1) and the most widespread immunotoxicity in humans (2). Immunotoxicity can be defined as adverse effects of the functioning of the immune system that result from exposure to chemical substances. Therefore, identifying chemicals that cause skin sensitisation is a key step in hazard identification and safety assessments of the ingredients contained in personal care products (3).

Skin sensitisation occurs in two main phases: the **induction** (or 'sensitisation') phase; and the **elicitation** phase (or 'response', upon re-exposure to the chemical sensitiser) (4). These two phases are comprehensively described below *(figure 2)*. The underlying molecular or cellular mechanistic steps (the adverse outcome pathway) that result in skin sensitisation are also described in the accompanying call-out boxes.

The **induction** (or 'sensitisation') phase begins when a skin-sensitising chemical is applied, once or repeatedly, to the surface of the skin and absorbed into the skin and subsequently into the systemic circulation. Initially, this contact causes local reactions in the skin, which then progress to a whole body (or systemic) reaction response (5). This may occur through an initial contact or multiple contacts over days or months. Skin sensitisation is a threshold phenomenon; hence, the scale of the local and systemic responses is dependent on the dose of the applied chemical per a specific skin area.

Next, the **elicitation** (or 'response') phase can occur. When the same substance comes into contact with skin again at a later stage (usually after a certain period, for example, a number of weeks) at a high enough concentration, and after a series of steps, inflammatory cells are recruited, resulting in an adverse skin reaction (5), known as the allergic contact dermatitis.

The challenge, therefore, in developing non-animal, alternative testing methods for skin sensitisation is to apply the mechanistic understanding of skin sensitisation to the design of predictive *in vitro*, *in chemico*, or *in silico* alternative testing methods (4).



In vivo describes a test performed or taking place in a whole living organism.

In vitro is a test performed in a test-tube, culture dish, or elsewhere (a controlled environment) outside a living organism.

In silico is the term given to a computer simulation of a test.

In chemico describes the use of chemical reactivity methods in testing.

Alternatives to animal testing for skin sensitisation

THE INDUCTION (OR 'TRIGGER') PHASE

The steps involved in the induction phase are as follows (5, 6) *(figure 2)*:

1. A skin-sensitising chemical, above a threshold amount, contacts and penetrates into and through the skin.

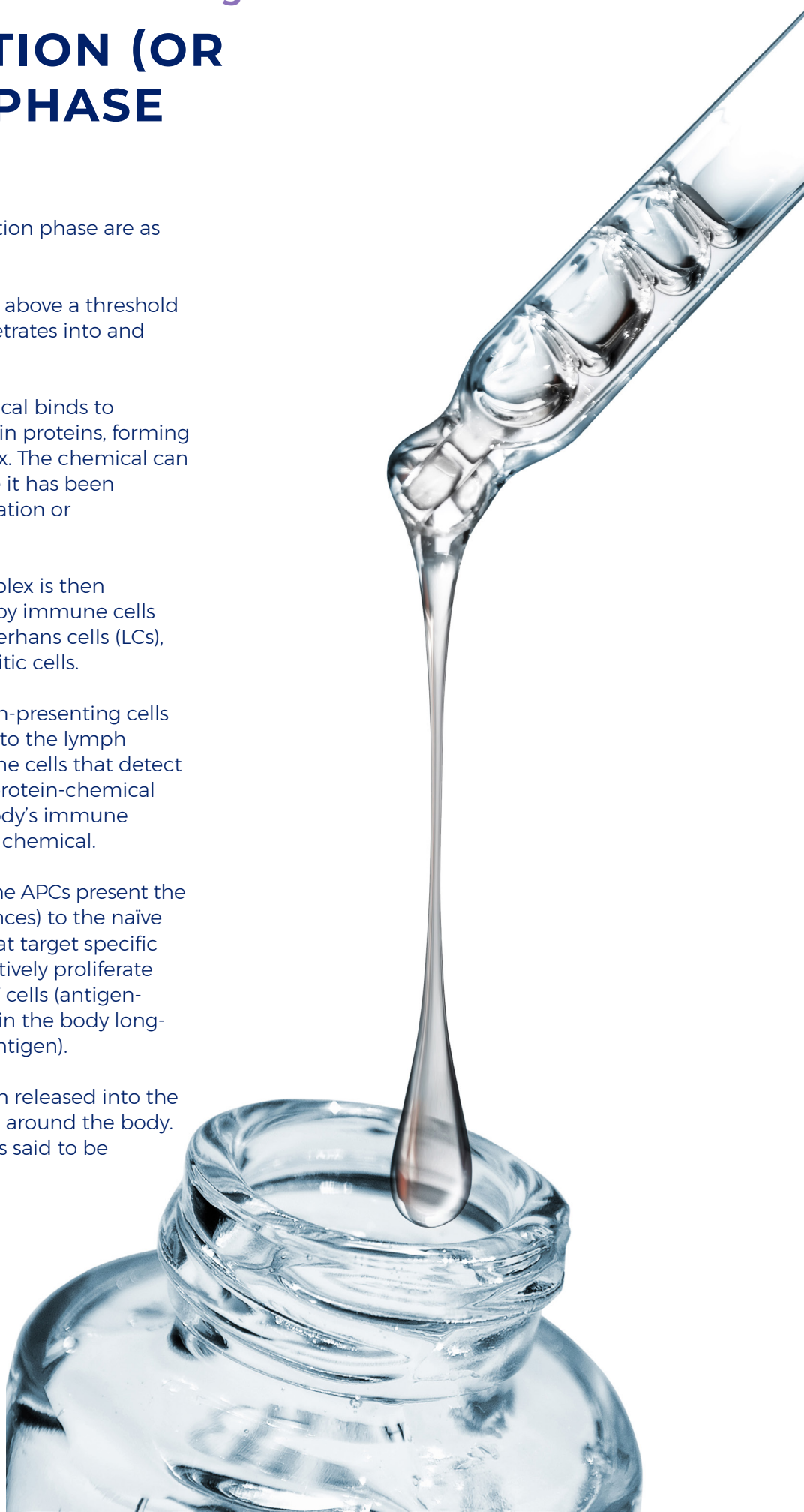
2. Once in the skin, the chemical binds to endogenous (or internal) skin proteins, forming a protein-chemical complex. The chemical can either bind directly, or once it has been activated, e.g. through oxidation or metabolism.

3. This protein-chemical complex is then recognised and processed by immune cells in the body known as Langerhans cells (LCs), which are immature dendritic cells.

4. The LCs mature into antigen-presenting cells (APCs) which then migrate to the lymph nodes. The APCs are immune cells that detect and engulf the processed protein-chemical complex and inform the body's immune response about the foreign chemical.

5. Once in the lymph nodes, the APCs present the antigens (or foreign substances) to the naïve T cells (white blood cells that target specific antigens), which then selectively proliferate (or multiply) into memory T cells (antigen-specific T cells that remain in the body long-term after removal of the antigen).

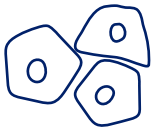
6. The memory T cells are then released into the blood circulation and travel around the body. At this point, an individual is said to be **sensitised**.



Alternatives to animal testing for skin sensitisation

THE ELICITATION (OR 'RESPONSE') PHASE

In response to secondary exposure of the chemical at sufficient levels, the same steps as in the induction phase will occur. When pre-programmed memory T cells encounter the foreign chemical and recognise it, pro-inflammatory molecules are released throughout the body, which recruit more T cells and trigger further production of pro-inflammatory molecules *(figure 2)*. This inflammatory response results in an adverse skin reaction that causes localised itching, burning, inflammation and redness of the skin, which is described as **allergic contact dermatitis (ACD)** (3, 5).



Langerhans cells (LCs) are a specific type of immune cell that form a network across the epidermis of the skin. They have the ability to migrate from the epidermis to the draining lymph nodes. Their location at the skin barrier affords them a key role as immune sentinels.

Dendritic cells are responsible for the initiation of adaptive immune responses and also function as sentinels of the immune system.

Figure 2. *The induction and elicitation phases of ACD in the skin.*

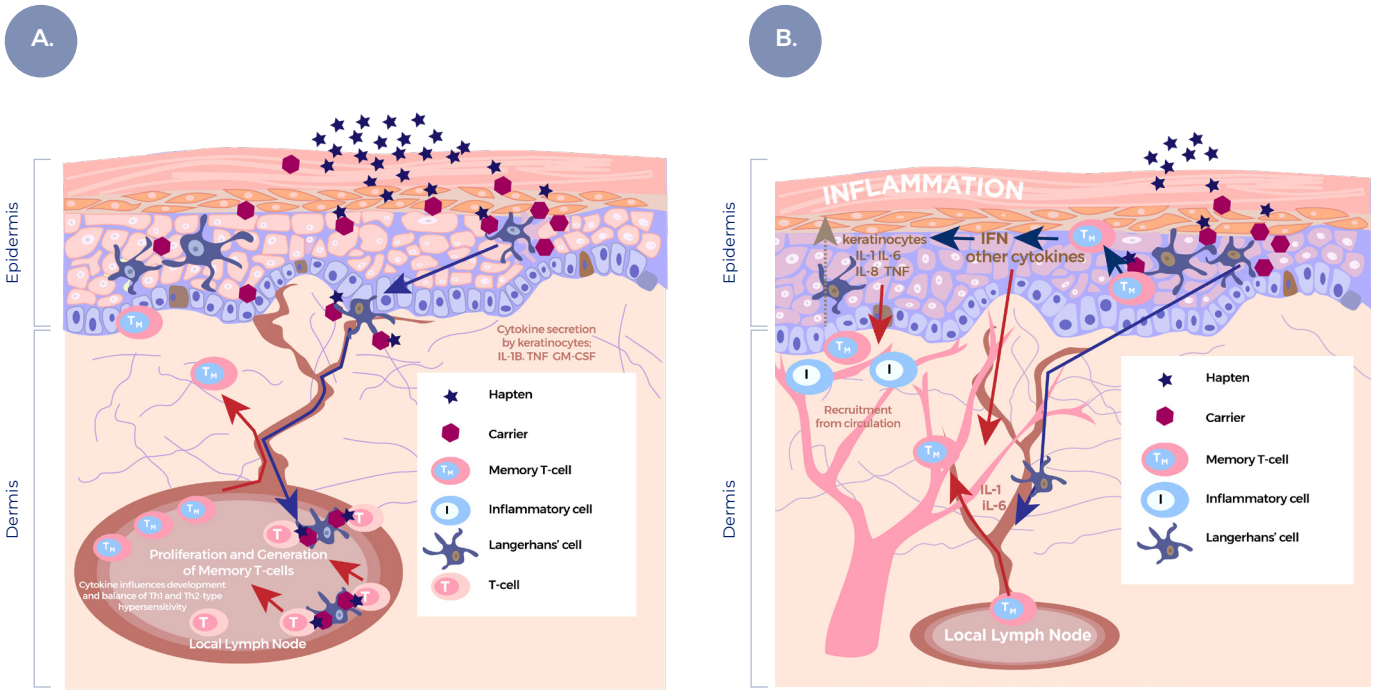


Figure adapted from reference: OECD 2014. A. The induction phase. B. The elicitation phase. ACD, allergic contact dermatitis; IFN, interferon; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumour necrosis factor.

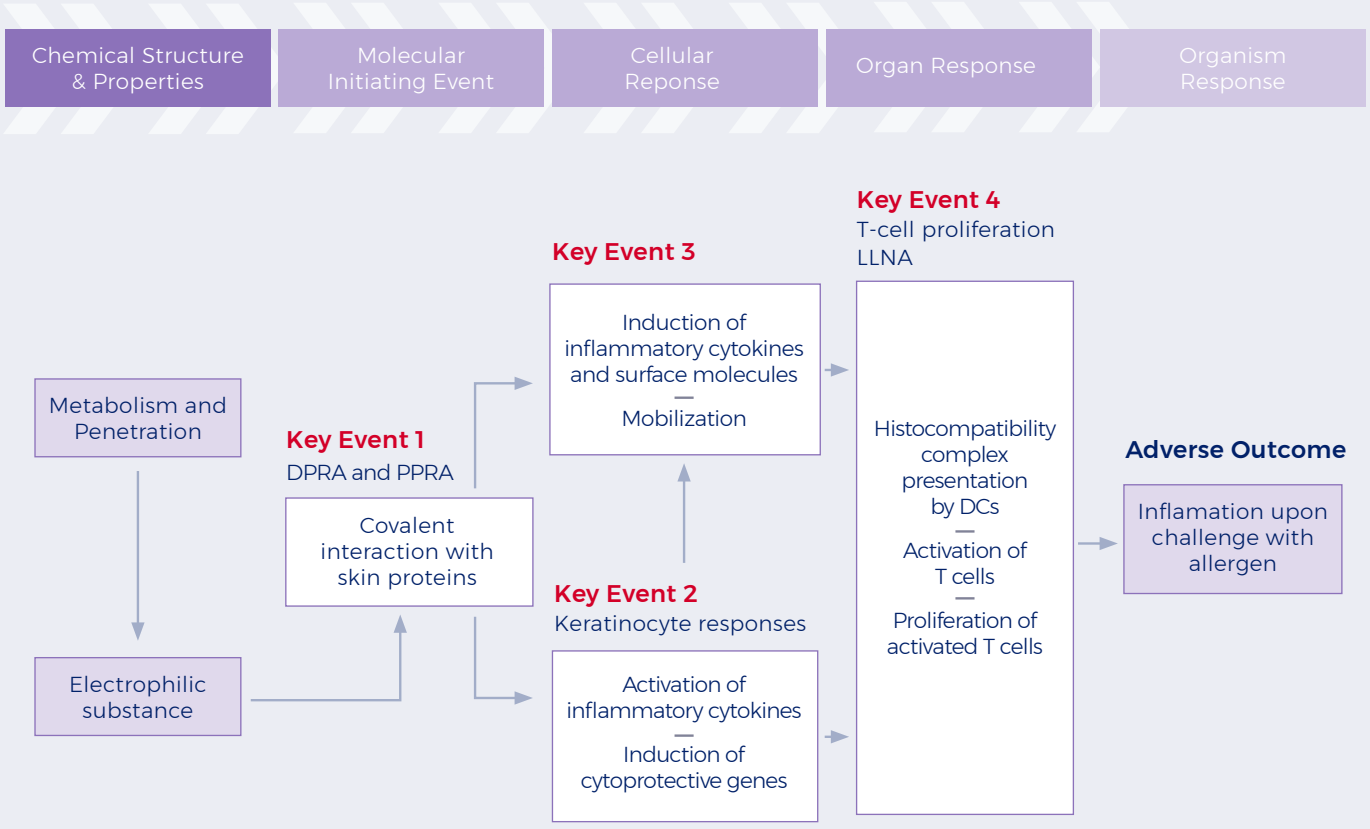
Alternatives to animal testing for skin sensitisation

THE ADVERSE OUTCOME PATHWAY (AOP) FOR SKIN SENSITISATION

In addition to increased efforts to **reduce** and **replace** animal testing methods, and the need to establish assay data as the 'standard' rather than relying on traditional animal testing, scientists have researched and developed a further pathway to be utilised when exploring skin sensitisation. This growing understanding has resulted in a clear picture of the steps involved in skin sensitisation, the **adverse outcome pathway** (AOP) (7, 8).

The AOP for skin sensitisation details the underlying cellular mechanistic steps that result in skin sensitisation (8). It is based upon the well-characterised pathways that drive both the induction and elicitation phases and represents a sequence of four observable and measurable key events that, in turn, track progression to skin sensitisation *(figure 3)*.

Figure 3. *The key stages of the AOP and the methods addressing these stages.*



Alternatives to animal testing for skin sensitisation

ASSESSING THE EFFECTS OF CHEMICALS ON SKIN

When assessing product safety, it is important to consider the induction phase while also informing consumers of possible elicitation responses. Both induction and elicitation phases occur at different thresholds of dose applied per area of skin.

The safety assessment of consumer products focuses on the avoidance of the induction phase. Therefore, the elicitation phase and the subsequent trigger of ACD will not occur, as the consumer is protected from the initial induction response.

Historically, assessing the induction phase was dependent on the **guinea pig maximisation test** (GPMT), an *in vivo* (in a living organism) testing method (9). To assess the effects of a chemical, this test involved administering multiple intra-dermal (skin) injections of the experimental chemical to guinea pigs; the injections also contained an adjuvant oil suspension (creating a stronger immune response) to ensure delivery of the chemical (9).

P&G **refined** the GPMT to develop another in vivo method using guinea pigs, known as the **Buehler test**, which was initially proposed in the 1960s, but modified in 1980. This test allowed for the direct topical exposure of chemicals (rather than using an injection), and could provide an initial indication of the sensitisation potential of the experimental chemical in question (9). For many years, the Buehler test and the GPMT were the preferred methods for skin sensitisation testing. These enabled a clear understanding of the effects of different chemicals when applied to skin.

In 1989, driven by the desire to **refine** and **reduce** the animal testing methods used in experimental research, P&G collaborated with Dr Ian Kimber's group to develop the local lymph node assay (LLNA) – a test used to evaluate skin sensitisation in mice (10).

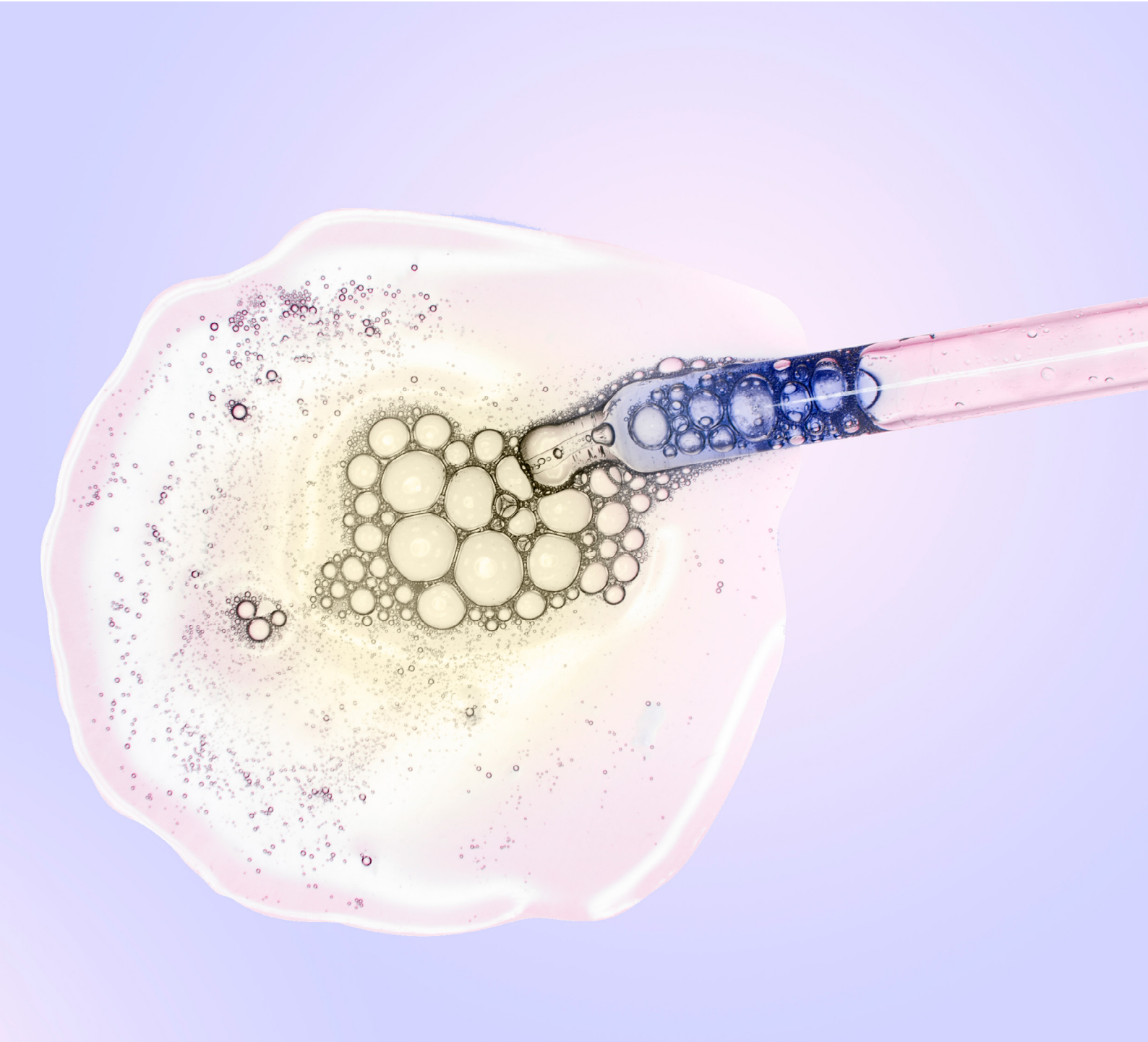
In the LLNA, the experimental chemical is applied to the ears of the mice (10). Chemical sensitisers induce the accumulation of lymphocytes in the local lymph nodes around the site of topical administration (10). In the LLNA test, the sensitisation potential of an ingredient is assessed by measuring the incorporation of radiolabelled thymidine into proliferating lymph node cells (LNCs) in a small number of animals (10, 11). This assay measures induction, in comparison to the GPMT which follows an ingredient throughout the entire immune process, resulting in ACD in the animals (9).

Despite still being an *in vivo* test method, the LLNA was optimised over subsequent years, resulting in its wide acceptance as a reliable and effective method of identifying contact allergens. Compared with the GPMT and the Buehler test, the LLNA uses fewer animals per treatment group, greatly reducing the amount of suffering caused (12).

The LLNA was a faster and cheaper method than the *in vivo* guinea pig testing models commonly used in the 1980s, and also provided information on sensitisation responses at different chemical exposures, rather than at a single dose (12). It provided more information of sensitisation potency, thus enabling a better safety assessment.

The Organisation for Economic Co-operation and Development (OECD) (13)

The OECD is an intergovernmental economic organisation comprising 38 member countries that promote policies to improve the economic and social well-being of people around the world. The OECD recognises the need to protect animals and encourages the uptake and use of non-animal-based testing methods. In 1981, the OECD introduced a set of internationally accepted specifications known as the OECD Guidelines for the Testing of Chemicals (OECD TG). The Buehler test was included as a test guideline (TG) 406 in 1981 and the LLNA was adopted by the OECD as TG 429 in 2002. The Direct Peptide Reactivity Assay (DPRA), a non-animal P&G assay described in detail below, was accepted by the OECD as TG 442C in 2015.



Alternatives to animal testing for skin sensitisation

DEVELOPING NEW APPROACH METHODOLOGIES (NAMS) TO REPLACE ANIMAL TESTING

To drive ongoing advances and increase non-animal testing options, researchers developed reference databases containing a set of chemicals that had been previously tested using the LLNA (10). These databases also include the physiochemical properties of these chemicals, their use categories and the functions that correspond to their potential to cause skin sensitisation (2, 3, 14).

In 2001, using these reference databases as a starting point, P&G focused on devising methodologies whose aim was to **replace** animal testing, rather than simply **refine** it, and to **reduce** the number of animals in tests. These new approach methodologies (NAM) included the development of *in vitro, in silico* and *in chemico* assays. These NAMs have become the preferred choice when generating new data for hazard identification and characterisation in skin sensitisation safety assessment.

Researchers had already begun looking into changes in surface markers or gene expression of immune cells following exposure to allergens. This research led to the development of cell-based *in vitro* methods for contact sensitisation, to understand the progressive amplification of a response to each test molecule (4, 15-20).

P&G has also collaborated with industry and academic partners to help in the development of a number of other methods of testing for sensitisation, such as the initial work on the **quantitative structure activity relationship** (QSAR)/ **tissue metabolism stimulator** (TIMES-SS) to predict skin sensitisation potency, incorporating both skin metabolism and chemical reaction patterns (21, 22). QSAR is a computational modelling method for revealing relationships between structural properties of chemical compounds and biological activities.

Alternatives to animal testing for skin sensitisation

P&G ASSAYS TO TEST FOR SKIN SENSITISATION

Driven by the goal to replace animal testing methods, P&G has taken an active role in developing non-animal-based skin sensitisation testing methods. Aside from the animal welfare benefits, non-animal testing methods can be faster, cheaper, and more accurate than typical animal-based methods.

The first assay developed by P&G was the **Direct Peptide Reactivity Assay** (DPRA); an *in chemico* method that tests chemical reactivity as a surrogate for skin sensitisation *(figure 4*). It was the first non-animal test to be evaluated in the European Union Reference Laboratory for Alternatives to Animal Testing-led validation study. The DPRA was also adopted by the OECD as Test No. 442C for skin sensitisation (23).

This simple, chemistry-based assay is considered a tool to be used in combination with other assays and addresses the first key event of the AOP of skin sensitisation *(figure 3)*, namely the way in which the chemical binds to skin proteins. The DPRA assay evaluates the reactivity potential of a chemical/antigen to proteins in the skin by mimicking this process with artificial peptides containing the amino acids lysine or cysteine (which both contain nucleophilic side chains that are known to react with electrophilic sensitising chemicals). The reactivity between the chemical antigen with the peptides is analysed through high-performance liquid chromatography (HPLC), a technique used to separate, identify and quantify different components in a mixture (23-25).

Measuring the depletion of an artificial peptide (containing cysteine or lysine), resulting from the binding of a test chemical to the peptide, is used in classification tree models to identify chemicals as sensitisers or non-sensitisers (26). In simple terms, the greater extent to which an ingredient reacts and depletes the artificial peptide, the more potent it may be to induce skin allergy. The DPRA has shown great promise for assisting in hazard identification and assessing skin sensitising potency when used in combination with other testing methods (27).

During skin sensitisation, certain ingredients can penetrate into the skin and directly react with skin proteins. Other ingredients require molecular transformation before they can react with these proteins.

The DPRA was not designed to assess ingredients that require metabolization by enzymes (metabolic activation) or abiotic transformation (through oxidation) before they become reactive to skin proteins.

Due to this limitation of the DPRA, P&G developed a 'next generation' assay for ingredients that are activated through metabolization or oxidation.

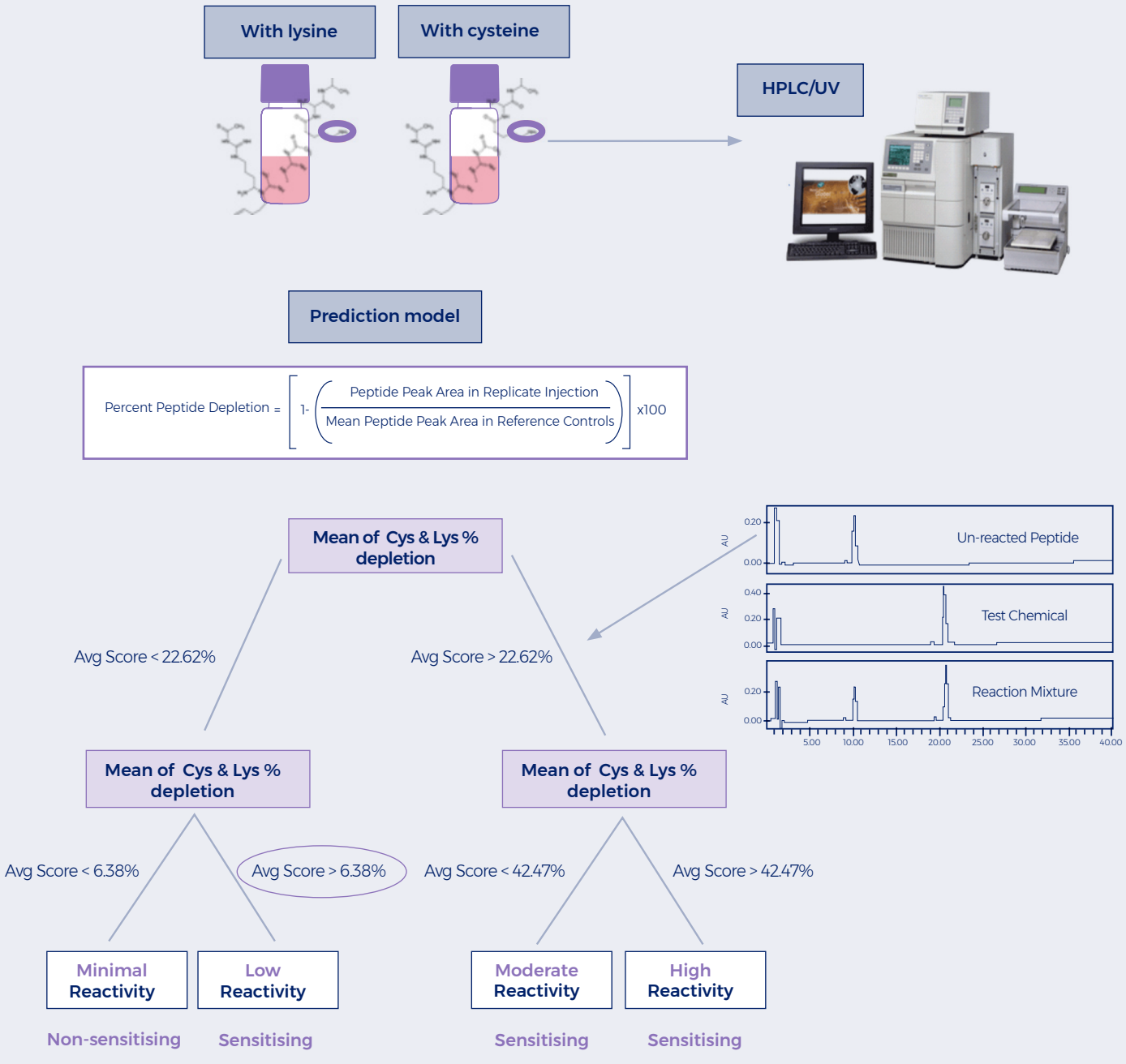
The **Peroxidase Peptide Reactivity Assay** (PPRA) was first described as a refinement of the DPRA (28, 29). This assay is performed with reactions containing the same cysteine- or lysine-containing synthetic peptides used in the DPRA (27). The PPRA incorporates dose-response analysis, mass spectrometry for peptide detection and a metabolism system that increases the potential of identifying the sensitising chemicals that cannot be assessed by DRPA (29). This means that the assay has a method of activating chemicals before assessing their reactivity. This technique has been refined over the years and is a reliable method of predicting the skin sensitisation potential of all chemicals (29).

Figure 4. *Three steps of a DPRA assessment.*

1. Incubate the test chemical/antigen with the synthetic model peptides (lysine and cysteine) for 24 hours.

2. Analyse chemical using HPLC and identify peaks.

3. Assess using a prediction model.



Combining non-animal approaches for predicting skin sensitisation potential of chemicals

DEFINED APPROACHES (DAs)

Individual methods alone, such as the DPRA or PPRA, are not sufficient to predict the skin sensitisation potential of a chemical. These methods address individual steps of the AOP, but not the whole pathway. Therefore, to ensure a more complete approach, there is a need to combine methods to predict skin sensitisation potential. This combination approach is carried out via **defined approaches** or DAs.

DAs use a fixed data integration procedure, combining NAMs, and possibly other data such as *in silico* information or physiochemical properties, to predict skin sensitisation. These DAs form part of the integrated approach on testing and assessment (IATA) of skin sensitisation (30).

DAs can be used alone to predict the skin sensitisation potential, or within a **Next Generation Risk Assessment** (NGRA) process (31). An NGRA is an exposure-led, hypothesis-driven risk assessment approach that integrates NAMs to ensure the safety of chemicals, without the use of animal methods (31).

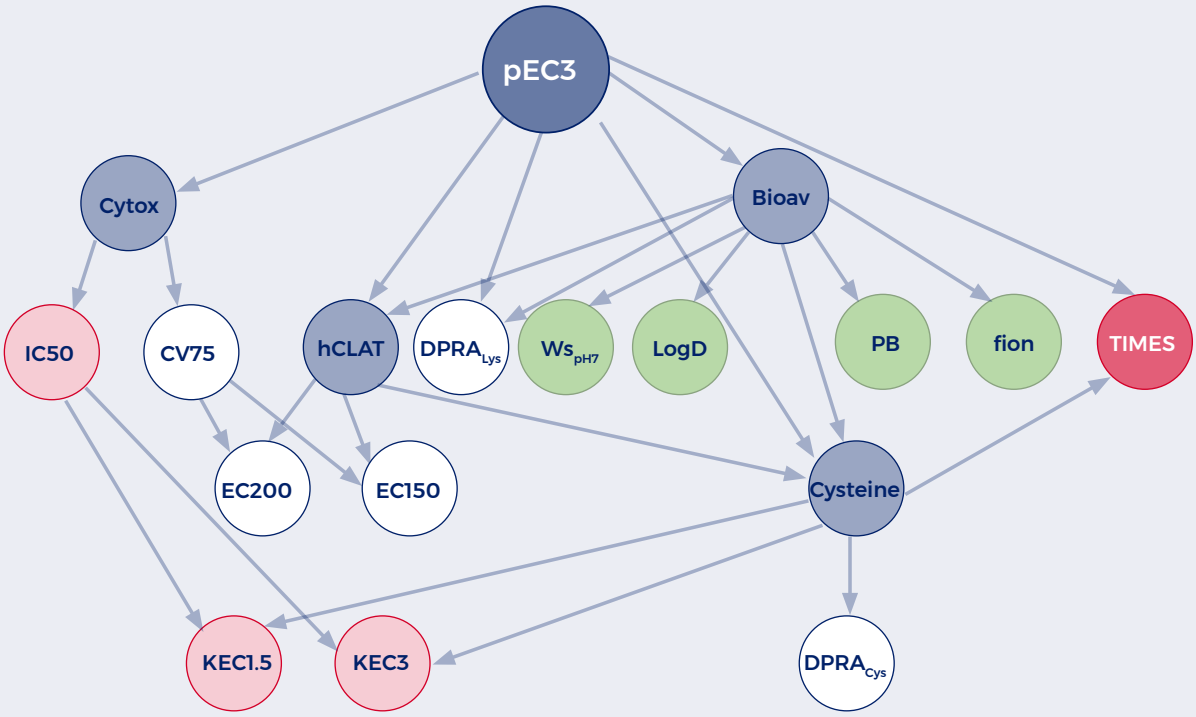
A broad assessment of DAs shows that all of the non-animal methods evaluated perform as well as, or better than, the LLNA in predicting human sensitisation endpoints for both hazard and potency (30). This means that DAs are superior safety methods, scientifically speaking, as they assess individual steps (30).

P&G has been an active contributor to many key industry collaborations, which have led to the publication of research papers, detailing data integration procedures to support regulatory decision making. Research has also contributed to the development of an NGRA approach.

One of these DAs, pioneered by P&G, used a **Bayesian network** (BN) approach *(figure 5)* (32, 33). This approach allows the prediction of skin sensitisation and also the estimation of potency, and is used in combination with NGRAs (32).

Combining non-animal approaches for predicting skin sensitisation potential of chemicals

P&G BAYESIAN NETWORK FRAMEWORK



The term Bayesian derives from the 18th century mathematician, Thomas Bayes, who provided the first mathematical treatment for statistical data analysis using what is known as Bayesian inference. A BN is a visual, probabilistic, graphical model that includes the use of standard probability distributions, allowing researchers to account for error or randomness in the statistical models used to analyse data. In this example BN, each node (or dot) represents a single model variable and each connection (edges or lines) between the two nodes represents how the variables influence each other and the final value (33, 34).

Figure 5. *Example of a Bayesian network.*

P&G's BN framework predicts the skin sensitisation hazard and potency of a chemical by considering the available NAM data. This includes *in silico* and physiochemical properties, and provides a probability and confidence interval in the prediction made (33).

The **BN approach** developed by P&G uses a class method to categorise chemicals into 4 potency groups. This approach ranges from C1 to C4 and corresponds to non-sensitiser, weak, moderate and strong sensitisers, and makes a prediction of the sensitisation potency for a chemical (33). This BN approach can be applied within the recently developed NGRA framework (31) and is considered a promising DA with high performance potential (33).

DAs have an important role to play in determining the hazard and potency of chemicals in humans using non-animal testing methods (33). Research in this area is currently active, diverse and is always evolving, as new and improved assays are being developed (35). It is important that research in this field continues to be developed and refined in order to progress this combination of techniques to develop further alternatives to animal testing.

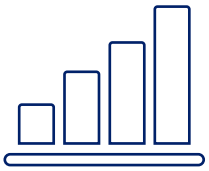
Bridging the gap with read-across: an alternative approach to animal testing

Combined efforts have led the personal care community to identify alternatives to animal testing for a number of key endpoints. However, there is still a long way to go. For example, systemic toxicity is one area where animal testing has not yet been completely replaced.

A systemic toxicant is a substance that can reach and affect many organs or the entire body rather than the site where it makes contact. For example, potassium cyanide is a systemic toxicant that affects virtually every organ in the body by interfering with cells' ability to utilise oxygen (36). Systemic toxicity also applies to potential effects of repeated, small dose exposures to substances. The systemic effects from repeated exposure to the same chemical remain difficult to understand and historically relied solely on animal testing (37).

Despite significant advances being made in recent years, a direct replacement to live animal testing has not yet been developed, but it is thought that the integration of NAMs is the likely way forward (37). However, since there is a lack of knowledge around handling, combining and interpreting new data from various NAMs, this integration of approaches requires further development (37). More importantly, there is also a lack of consistency in the data requirements of the various regulatory agencies around the world, which has led to ambiguity in the wider implementation of methods in regulatory toxicology (37).

Due to the complexity of human biology, it is unlikely that one, single, stand-alone test or method will ever completely replace animal testing. A comprehensive approach, using a combination of lab-based methods that encompass *in vitro, in silico,* and omics data, is the likely more effective means of addressing systemic toxicological endpoints in humans (38). Ongoing progress in the field of non-animal testing depends on the thorough review, validation and acceptance of testing strategies and protocols by regulatory agencies across the world (38).



Omics data: analysis of large amounts of data representing an entire set of information, especially the entire set of molecules, such as genes, proteins, lipids, or metabolites, in a cell, organ, or organism.

Research in various areas, such as **read-across** (a technique for predicting endpoint information for a target substance by using data from another similar substance), has led to a structured combination of methods to foster further alternatives to animal testing. These methods combine both pre-existing data, from animal tests on structurally or biologically similar chemicals, and data from *in vitro* and *in silico* methods to assess toxicological endpoints. The results obtained help to make a prediction about the effects of chemicals that have not been tested on animals.

Read-across: Using computational power to predict the safety of ingredients

The historical method of assessing human safety risk using data from animal testing is no longer the only way to identify possible hazards. As described in the skin sensitisation section above, a number of methods have **refined, reduced**, and even **replaced** the need for animal testing, but there are still data gaps for many chemicals that need to be evaluated.

One recent approach to assess chemicals with little or no direct safety data has led to the development of predictive toxicology, namely **Structure Activity Relationship** (SAR)-based read-across. This is based on the hypothesis that the biological activity of a chemical is a direct result of its molecular conformation (39). Therefore, chemicals with similar structures are expected to react in a similar manner and elicit similar toxicological responses (39).

Over the last decade, P&G has led the way in research for read-across in a variety of areas, including source chemical identification and evaluation, uncertainty evaluation, case studies and NAMs to enhance predictivity (39-43). The framework to identify source chemicals, outlining the use of expert judgement to assess similarities in chemical structure, reactivity, metabolism and physiochemical properties, was set forward by P&G, and more consistent and transparent rating 'rules' have been developed for several compound categories (42). The framework and concepts have been accepted and adapted by many other organisations, including global regulatory bodies (39).

Bridging the gap with read-across: an alternative approach to animal testing

WHAT IS READ-ACROSS?

Read-across is a process whereby existing safety endpoint information for one chemical (the source chemical) is used to predict that for another chemical (the target chemical) (40). These chemicals are considered to be 'similar' molecules; usually on the basis of structural similarity, metabolic similarity and/or on the basis of the same mode or mechanisms of action (37, 39, 44).

The **target chemical** (also known as the 'structure of interest') is the 'chemical of interest' (for instance, a newly devised ingredient) which lacks direct data, and therefore has a 'data gap' for an endpoint.

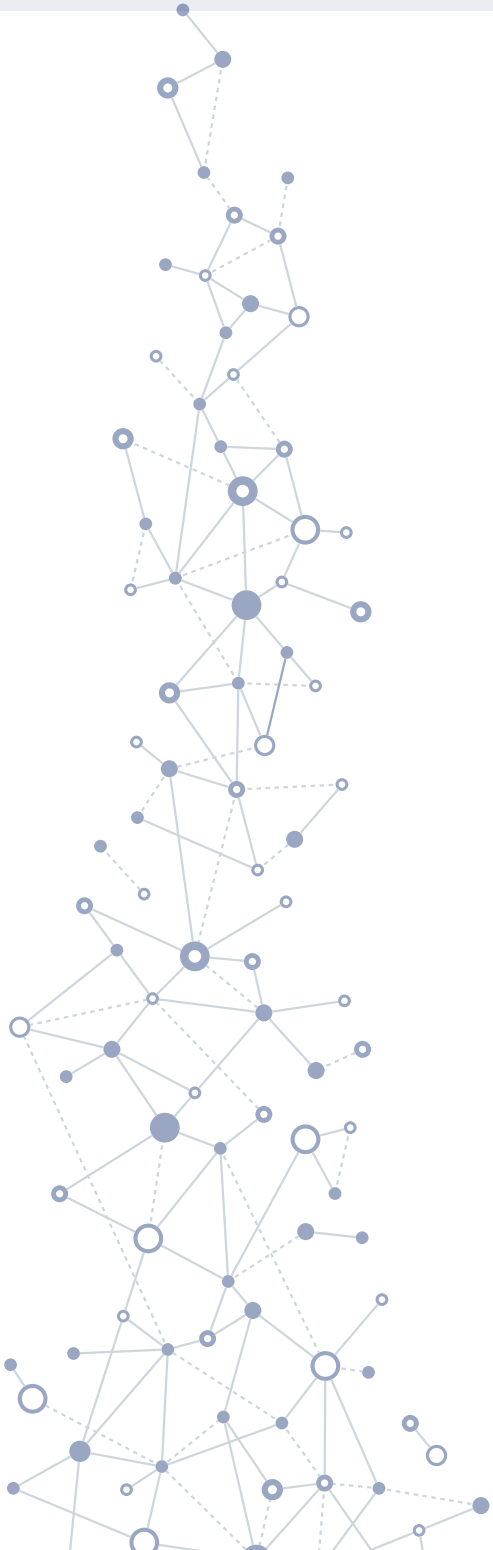
The **source chemical** (also called 'analogue') is the chemical with existing direct data and is 'data rich' for an endpoint. This source chemical is used as a model to fill the data gaps for the specific target chemical (37, 39, 44).

In a read-across assessment, different approaches are used depending upon the source chemical and target chemical. Analogue approaches focus on single or limited chemicals, whereas category approaches focus on a group of structurally related chemicals (i.e. analogues) that are considered similar to the target chemical. The physiochemical and toxicological properties of the source chemicals are likely to follow a regular pattern as a result of structural similarity, and hence have similar biochemical pathways and metabolism. As these chemicals are analysed, structural differences between chemicals often reveal variances in physiochemical properties, reactivity, absorption, distribution, metabolism and excretion (39). Therefore, toxicokinetics and toxicodynamics information should be included when assessing chemicals with either read-across approaches (39).

The results of a read-across assessment are expected to offer a similar level of confidence that direct data would usually provide (39). Read-across is considered an acceptable alternative to animal testing by most regulatory agencies, including the European Chemicals Agency (44), Health Canada (45) and the U.S. Environmental Protection Agency (39, 46). Read-across has also become important as an alternative to animal testing in many industries, such as high-production volume (HPV) chemicals, food additives, fragrances and cosmetic ingredients (40, 41).

What is a Point of Departure (POD)?

In toxicology, a POD is defined as the point on a toxicological dose-response curve established from experimental data or observational data, generally corresponding to an estimated low effect level or no effect level (also known as no observed effect level [NOEL] or no observed adverse effect level [NOAEL]) (47). The POD marks the beginning of extrapolation to human toxicological reference dose or reference concentration (eg. Acceptable Daily Intake). The POD is at least 100 fold higher than the maximum exposure of an ingredient which is assessed to be safe for humans.



Toxicokinetics describes how a substance is absorbed into the body and what happens to it in the body thereafter – what the body does to a chemical and how it is removed from the body.

Toxicodynamics is a quantitative description of the effects of a toxicant on a biological system – what a chemical does to the body. These effects include a range of endpoints and products, ranging from the molecular level, to cells, tissues, organ systems, and life-history traits.

Physicochemical properties include both physical and chemical characteristics of a substance, including solubility, melting point, viscosity, and more, and can impact the toxicokinetic and toxicodynamic properties of a chemical.

Bridging the gap with read-across: an alternative approach to animal testing

WHAT STEPS ARE INVOLVED IN A READ-ACROSS ASSESSMENT?

A SAR-based read-across assessment is a complicated, rigorous, iterative process. A **weight of evidence** (WOE) approach is used to evaluate and integrate multiple data streams to support the read-across hypothesis *(figure 6)*.

Problem formulation and defining the decision context are the first steps in performing a read-across assessment, followed by the development of the read-across hypothesis (37). Once the read-across assessment is complete, the data are used to write a read-across justification document, describing the assessments carried out in the read-across.

This justification document provides the scientific rationale and evidence to support the hypothesis, and explains why the read-across extrapolation of data from a source chemical to the target chemical is scientifically appropriate. In addition, areas of uncertainty for each scientific element should be identified (39). Finally, the read-across conclusion can be made, dependent on the decision context and the uncertainties.

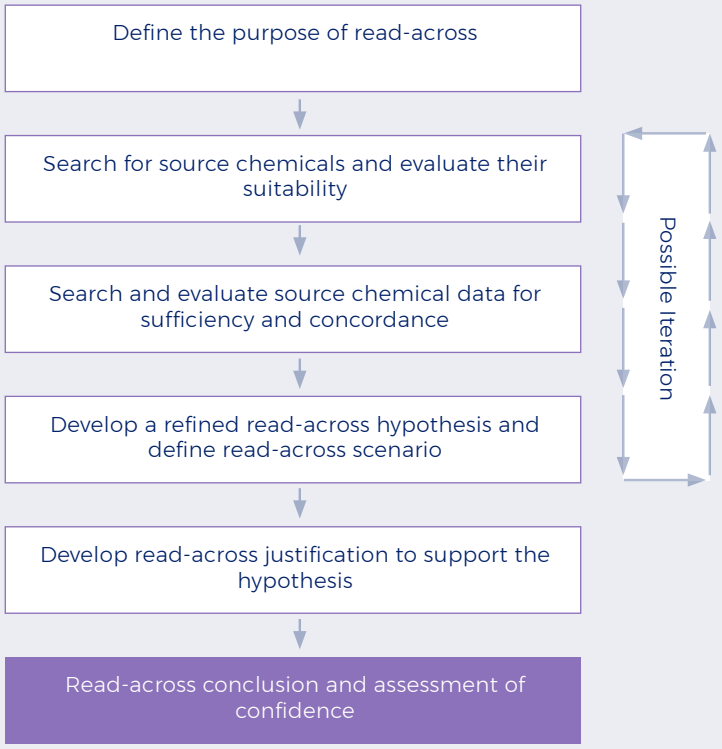


Figure 6: *Flow chart showing the steps of read-across.*

1. Step one: Define the purpose of read-across

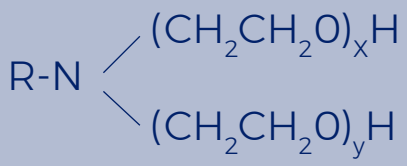
The first step involves describing the purpose of the read-across, occasionally referred to as 'problem formulation'. This step includes searching for and reviewing all relevant toxicological data and information to identify the data gaps that exist for the target chemical, and the context of how these decisions were made. This is a description of how the read-across will be used. It may be for prioritisation or screening purposes (to compare one chemical to another), to decide which chemicals require further assessment or for a hazard assessment (40). Read-across may also be used to identify a POD for chemical registration, classification and labelling, as well as for a human health risk assessment.

A number of publicly available data sources exist, for example, to help identify data gaps, PubMed, ACToR and eChemPortal. Data obtained from different sources can provide information on physiochemical properties, observed effects and potency on different endpoints in toxicological studies and Absorption, Distribution, Metabolism and Excretion (ADME) (40).

PEG cocamines for personal care products

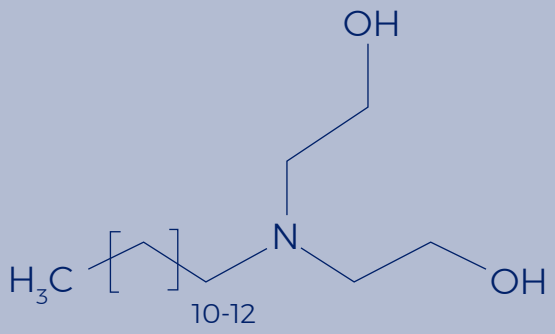
SAR read-across was used for a human health risk assessment of polyethylene glycol (PEG) cocamines, a surfactant that can function as an emulsifying and solubilising agent. In personal care products, PEG cocamines help to form emulsions by reducing the surface tension of the substances and help ingredients dissolve in a substance where they would normally not dissolve. The data surrounding PEG cocamines were found to be insufficient to support a safety assessment of PEG cocamine ingredients. Among the data gaps identified, repeated dose toxicity data on PEG-2 cocamine were needed to demonstrate that relevant exposure to the ingredient with the lowest molecular weight in this group would be considered safe for its intended use.

PEG cocamines listed in the International Cosmetic Ingredient Dictionary include PEG-2, PEG-3, PEG-4, PEG-5, PEG-8, PEG-10, PEG-12, PEG-15 and PEG-20 cocamine. This is the basic structure of PEG cocamines:



R represents alkyl groups from fatty acids, and the x+y of the polyethylene groups have average values equal to the number in their name (for example, in PEG-4, x+y has an average value of 4).

For the purpose of this case study, based on Skare et al, 2015, PEG-2 has been selected as the target chemical. The structure of PEG-2 is:



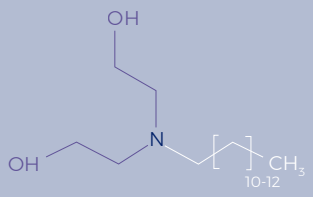
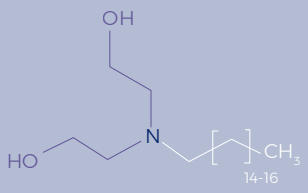
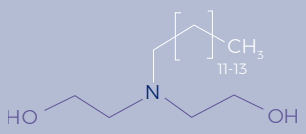
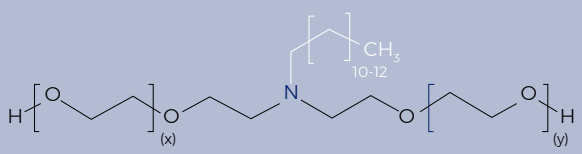
2. Step two: Search for source chemicals and evaluate their suitability

This step includes evaluating the structural features of the target chemical in order to identify appropriate potential source chemicals. This is based on the initial hypothesis that chemicals with structural similarities will produce similar biological effects (39). Chemicals with similar structures, such as a common functional group, can be identified using publicly available tools, such as ChemIDplus and QSAR toolbox.

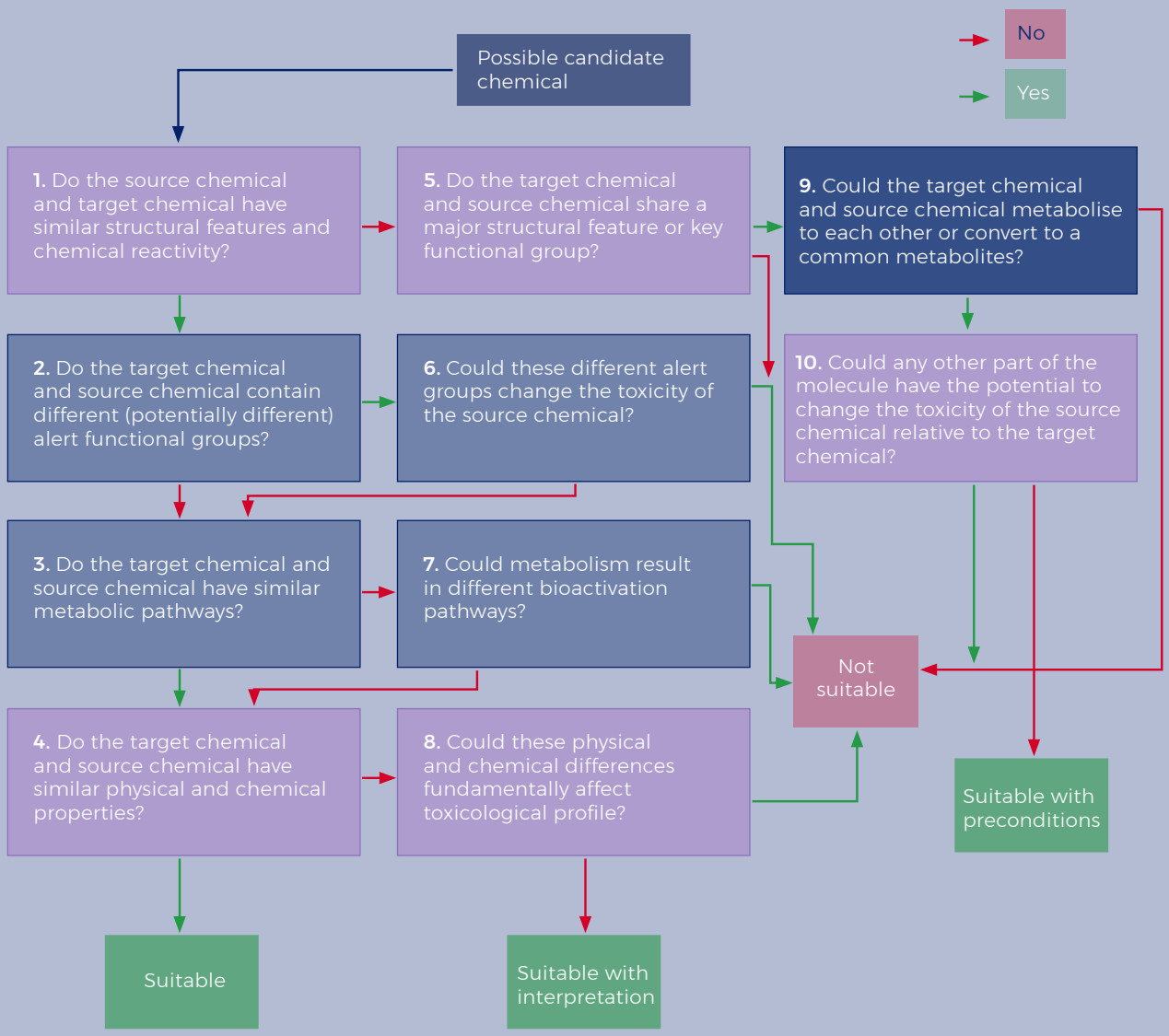
The most appropriate and suitable source chemicals must include features beyond structural similarity. They could include specific physiochemical properties that could impact bioavailability, the metabolic pathway, common breakdown products, *in silico* structural alerts and other QSAR evaluations, compared with the target chemical (42). In 2010, a decision tree-based framework developed by P&G to guide the source chemical suitability evaluation was the first time taking all above mentioned features into consideration. This framework has been widely accepted and adapted by many others (41, 42).

Which chemicals have similarity to PEG-2 cocamine?

Using publicly available tools, three chemicals with structural similarity to PEG-2 cocamine were identified.

	Representative structure for SAR	***Common name***
Target		**PEG-2 cocamine**
Source		**PEG-2 tallow amine**
Source		**PEG-2 C13-15 alkyl amine**
Source		**PEG-4 cocamine**

A decision tree, developed by P&G, was then used to assess the suitability of the identified chemicals for use in the read-across assessment:



Using this decision tree, **PEG-2 tallow amine** and **PEG-2 C13-15 alkyl amine** were found to be **suitable** based on the same structural features and similar physiochemical properties and metabolic pathways with the target chemical, PEG-2 cocamine. **PEG-4 cocamine** was found to be **suitable with interpretation** based on some differences on physiochemical properties and metabolism that do not significantly affect chemical reactivity in a way that would impact toxicology.

3. Step three: Search and evaluate source chemical data for sufficiency and concordance

The next step involves searching for, identifying and reviewing all relevant toxicological data and information for the identified source chemical. These data should then be analysed and evaluated for consistency and concordance across the target and source chemicals. The findings should also further explain the trends in the data or the clustering of effects that may parallel the variation in structural features between the chemicals. This is especially important for a category approach (41).

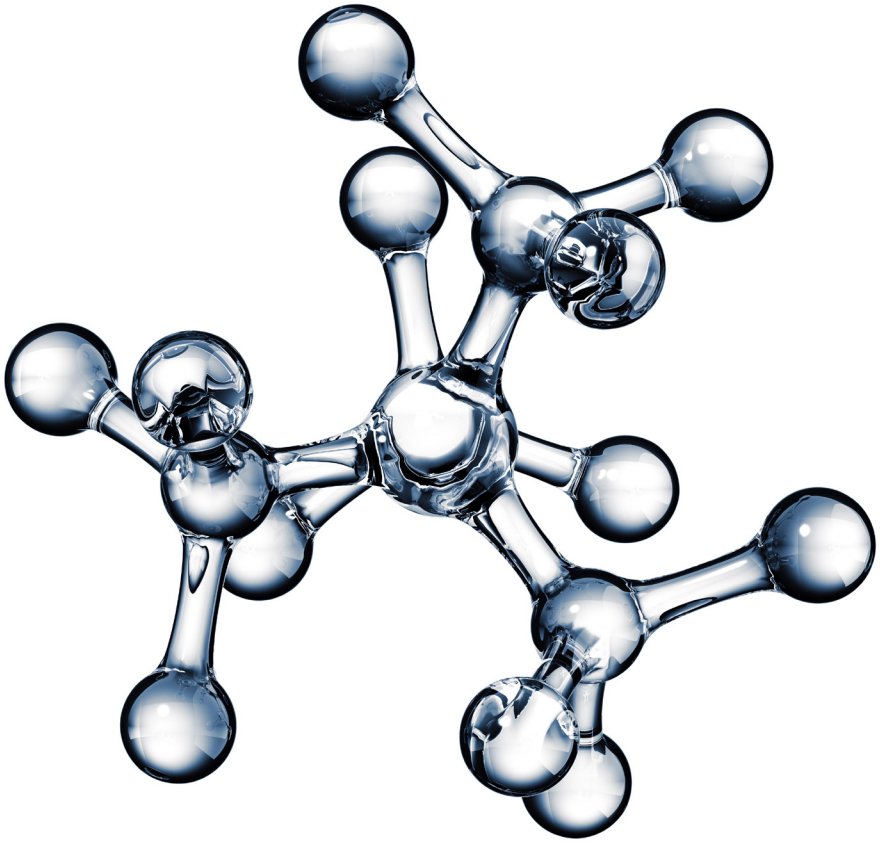
In addition to examining the data for consistency, it is important to determine any potency trends, and whether the dose at which a critical effect occurs, follows a predictable pattern (44). This should, in turn, align with a chemical structural change that occurs in both the target chemical and the source chemical(s) (39, 48).

What are the available data for source chemicals?

Databases were searched to find all the relevant data for the chemicals identified to be either suitable, suitable with interpretation or suitable with precondition. Data on metabolism and other toxicokinetic and toxicological effects in different endpoints and mechanisms of action were all considered to fill data gaps around PEG-2 cocamine repeated dose toxicity. Available repeated toxicity data were also identified for the two suitable source chemicals.

Chemical	Source chemical suitability	Study Type	POD	Effects observed
PEG-2 tallow amine	Suitable	Rat 90-day oral administration via diet	NOEL = 50 mg/kg/day	**Macroscopic observations:** yellow colouration and thickening of mucosa in small intestine and regional mesenteric lymph nodes at 450 mg/kg/day **Microscopic observations:** engorgement of villi and lamina propria of small intestine; swollen foamy macrophages in small intestine, Peyer's patches and regional lymph nodes at 150 and 450 mg/kg/day.
		Rat 90-day administration via diet	NOEL = 12 mg/kg/day	**Microscopic observations:** aggregation of foamy macrophages in small intestine and mesenteric lymph nodes at 400 mg/kg/day.
		Dog 90-day administration via diet	NOEL = 13 mg/kg/day	**Microscopic observations:** increased foamy macrophages in small intestine and regional lymph nodes at 40 and 120 mg/kg/day.
PEG-2 C13-15 alkyl amine	Suitable	Rat 90-day oral administration via gavage	NOAEL = 15 mg/kg/day	**Macro-** and **microscopic** changes in non-glandular stomach at 30 and 150 mg/kg/day; cataracts at 150 mg/kg/day
		Dog 90-day oral administration via capsule	NOAEL = 30 mg/kg/day	GI clinical signs at 100 mg/kg/day; **Microscopic observations:** increased pigment accumulation in Kupffer cells and bile canaliculi in females at 100 mg/kg/day

The data showed concordance and consistency between different studies for the same source chemical, and between two different source chemicals on toxicological effects observed (gastrointestinal tract effects) as well as on toxicity potency (POD, between 10-50 mg/kg/day). Data on genotoxicity and developmental and reproductive toxicity were also obtained (not shown here) and found to be similar between the source chemicals which provide further evidence on data concordance.



4. Step four: Develop a refined read-across hypothesis and define the read-across scenario

The initial evaluation of the source chemical(s), outlined in steps two and three, provides insight into the biological similarity of the source chemicals. This allows for the refinement of the initial read-across hypothesis, based on chemical structure, to a broader hypothesis, building on both the structural similarity and the biological similarity (44). Based on the evaluation of toxicological information on the source chemicals and an evaluation of metabolism information, the read-across hypothesis and scenario are developed. One example is discussed below:

What happens if the source chemical(s) show toxicity?

Chemical A, chemical B, and chemical C were evaluated as source chemicals for **chemical X**, the target chemical. Available information indicates that the target chemical is metabolised to an active metabolite that results in liver toxicity, similar to the source chemicals. In this scenario, it would be particularly important to develop a WOE that elucidates any potential differences in metabolism between the target chemical and source chemicals in the read-across.

Evaluation of source chemicals demonstrates that they cause liver toxicity

Evaluation of metabolism information demonstrated that the source chemicals undergo biotransformation to an active metabolite that is a known liver toxicant

Predictive metabolism information on the target chemical suggests that it would be similarly biotransformed to produce the same active metabolite

In this example, the read-across hypothesis and scenario would be that the target chemical **(chemical X)** will be metabolised to an active metabolite that will result in liver toxicity in a similar way to the source chemicals **(chemical A, chemical B, and chemical C).**

5. Step five: Develop read-across justification to support the hypothesis

The penultimate step involves creating the read-across justification to support the hypothesis and the scenario selected in the previous step.

The **justification** is a document that brings all data used in the read-across together, making a coherent and logical argument supporting the hypothesis (49). If there are data inconsistencies or a logical read-across argument cannot be made, then preceding steps would need to be revisited and a revised hypothesis would need to be made.

No read-across justification is identical to another (49). This is due to the nature of substances and chemical classes, and that the chemicals can be grouped in a variety of categories, such as, structural similarity, common toxicological and common physiochemical properties (49).

In addition to detailing the scientific robustness of the read-across, the justification document should also describe any areas of uncertainty (50). Uncertainties discovered at this point must be addressed and may lead to changes in the source chemical(s) used to provide data for the read-across assessment (50). This may lead to continued iterations of the previous steps, until suitable source chemicals are obtained (40). The goal is to establish the most scientifically accurate justification to support a read-across hypothesis with the highest level of confidence (50).

The justification document needs to include a comprehensive review and evaluation of the available information surrounding the target chemical and source chemicals (39, 44). This includes any in vivo toxicological data and should fully inform the biological similarity of the chemicals and the appropriateness of the read-across. Despite read-across justifications not being identical for each chemical, there are a number of key scientific elements that are considered and typically documented *(figure 7)*.

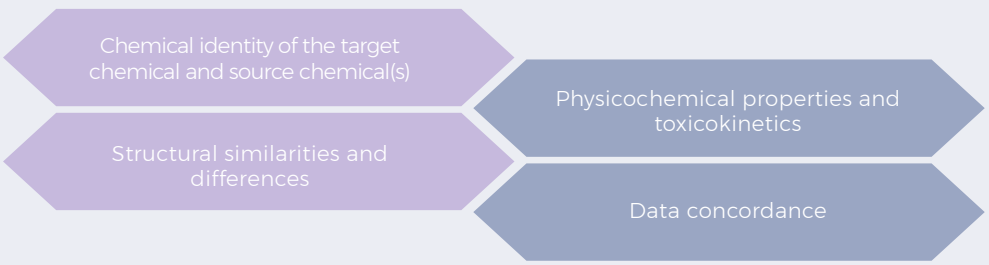


Figure 7. *Key scientific elements included in the justification document that are considered when performing a hazard assessment based on read-across.*

Chemical identity of the target chemical and source chemical(s)

The justification document should detail chemical structure, composition and impurities for both the target chemical and the source chemical(s) (44). For example, any known impurities should be clearly described, as differences in impurities may impact the toxicological profiles of the target chemical and source chemical(s) in different ways (51). This may impact the final prediction made about the target chemical.

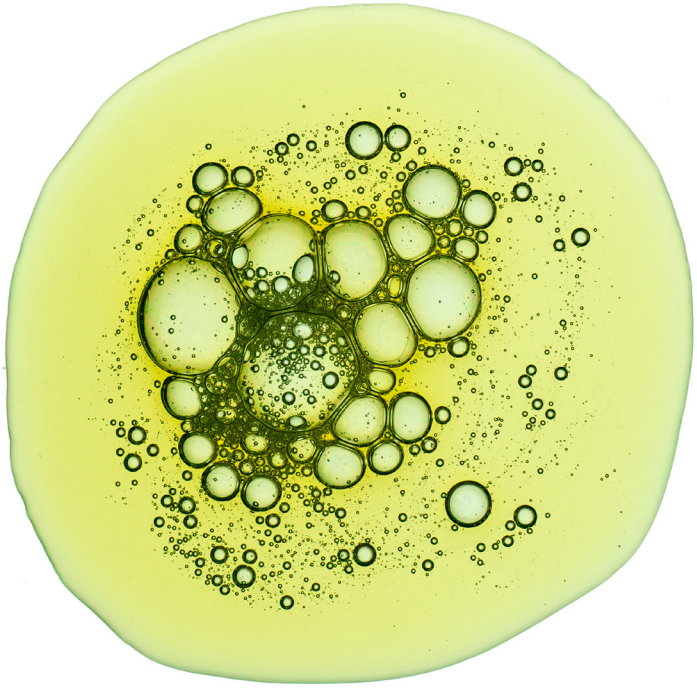
Structural similarities and differences

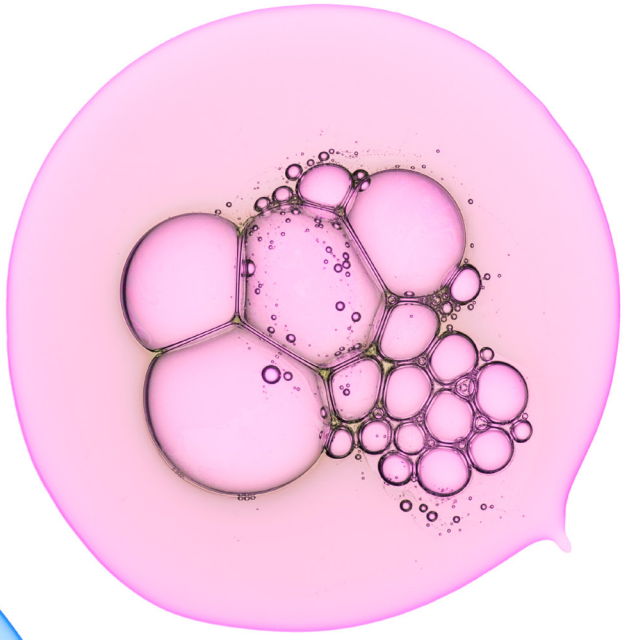
Understanding of the structural similarities between the target chemical and source chemicals could result in either equivalent biological activity or lack of biological effects (51). This may involve the core structure of the chemical, shared functional groups or common breakdown products. In addition to structural similarities, structural differences should be noted, along with explanations suggesting why these differences are not believed relevant to biological differences.

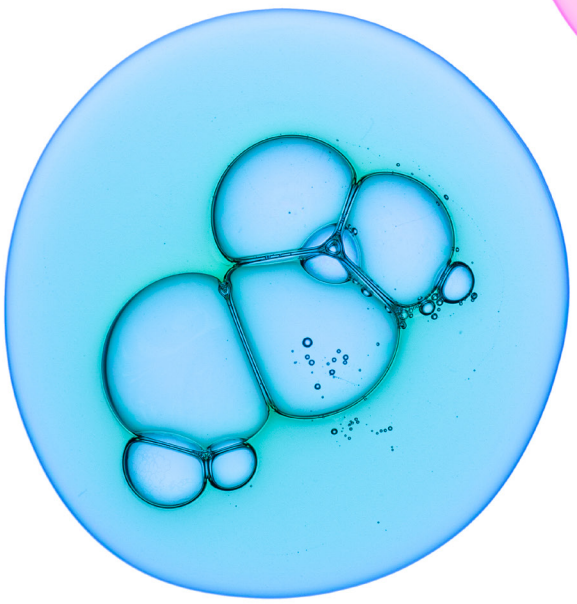
Physiochemical properties and toxicokinetics

Based on structural similarity, the target chemical and source chemical(s) may be expected to share similar physiochemical and toxicokinetic properties. These similarities would result in very similar exposures to any target organs by the target chemical and source chemical(s), and would cause similar effects and potency across the category members. However, the possibility exists that toxicokinetics may not be identical between the source and target chemical. Therefore, differences in the exposure to potential target organs could occur.

For example, biotransformation may not be identical between the source chemical(s) and target chemical, and exposure to non-common metabolites could occur. Therefore, it is important that the read-across justification addresses the potential toxicological impact on any non-common metabolites that might be formed between the target chemical and source chemical(s).







Biotransformation is the process by which one chemical changes to another within the body; this may involve any type of molecular reaction favoured physically or under enzymatic control. The body biotransforms chemicals in different ways. Metabolism occurs in some cases to make a molecule more water soluble so it can be excreted in the urine, or to detoxify it to a less reactive molecule that does not react with biological components, such as DNA or proteins.

Data concordance

This is the most critical aspect of a category approach read-across assessment. When data have been collected, it is useful to categorise them to facilitate their evaluation. Concordance of the primary critical biological effects across category members supports the conclusion of similar toxicodynamics across the category (43). For the endpoint that is being read-across, concordance of the source chemical data is important in order to secure a high confidence, low uncertainty read-across.

6. Step six: Read-across conclusion and assessment of confidence

The last step includes making a judgement based on the plausibility of the read-across hypothesis being true and the WOE provided in the read-across justification. At this stage, an uncertainty evaluation should be conducted (39, 50). In 2014, P&G published a framework to facilitate consistent characterisation of read-across uncertainty and increase transparency (39, 50). This framework emphasised that consideration must be explicitly given to the areas of uncertainty in the dataset or supporting information, and the impact of that uncertainty on the read-across. In addition, any limitations, adjustments or conditions for use of the read-across conclusion for the decision context established in step 1 should be described.

Read-across conclusion for PEG-2 cocamine

Once the justification document has been written with all the findings of the previous steps, the last section should detail the confidence in the observations made. A potential question concerning the use of read-across in risk assessments for systemic toxicity is the additional uncertainty introduced by extrapolation of source chemical data to the target chemical. By applying the criteria in the framework proposed by P&G (50) uncertainty in the read-across should be evaluated based on the scientific elements described in step 5. A reasonable read-across uncertainty factor for use in the quantitative risk assessment should be applied. In this example, the two suitable source chemicals share similar structure, physicochemical properties and metabolic pathway. The available high quality data consistently demonstrates similar toxicological effects and potency for this group of chemicals; the read-across for PEG cocamines would have a low uncertainty ranking and a default read-across uncertainty factor of 1 would be assigned. Therefore, this SAR-based read-across is considered robust and can conclude that PEG-2 cocamine would not be expected to result in repeated dose effects due to exposure when used in cosmetic products. This case study led by P&G has been accepted and adopted by the Cosmetic Ingredient Review expert panel for supporting the safe use of PEG cocamines in cosmetic product (52).

Many scientists today, including those at P&G, are looking at ways to improve read-across methods by employing NAMs data to support hypotheses with empirical non-animal data (43, 48, 53). The advancement of NAMs, such as high-throughput biological assays, *in vitro* tests, omics technologies and *in silico* prediction models provides additional opportunities for the improvement of read-across assessments.

NAM data can be used to make comparisons of biological activity, for exploring potential modes of action across related chemicals and to better understand metabolism and kinetics of a chemical. The inclusion of NAM data into the read-across to support or confirm biological similarity, and inform on potency differences, can significantly increase confidence and reduce uncertainty in a read-across (43). For these reasons, NAM-supported read-across is gaining popularity and is expected to grow as more types of NAM data streams become available in the future.

WORKING TOWARDS A FUTURE FREE FROM ANIMAL TESTING

P&G is striving to work towards a world without animal testing. Nonetheless, we understand that there is still much work to be done to achieve this goal. Combining the approaches discussed above to evaluate chemicals for personal care products will result in fewer animals being used in safety tests, for other products and ingredients. However, to operate in a world entirely without animal testing, it is important that all regulatory agencies accept and acknowledge the validity of non-animal safety methods.

P&G continues to collaborate with leading international animal welfare organisations, academia, industry associations and policy makers to help drive this change. Continual investment into research, such as in skin sensitisation, and approaches such as read-across, will contribute to the development of new alternative methods to animal testing and the onward scientific growth in this important field.

Working towards this central goal with organisations across the globe will continue to be a priority for P&G and we look forward to the day, in the not too distant future, when non-animal alternatives fully take over and animal testing for all kinds of products is a thing of the past.

REFERENCES

1. Gilmour N, Kimber I, Williams J, Maxwell G. Skin sensitisation: Uncertainties, challenges and opportunities for improved risk assessment. Contact Dermatitis. 2019;80:195-200.

2. Gerberick G, Vassallo J, Bailey R, Chaney J, Morrall S, Lepoittevin J. Development of a peptide reactivity assay for screening contact allergens Advance access publication. 2004;81:12.

3. Hoffmann S, Kleinstruer N, Alepee N, Allen D, Api A, Ashikaga T, et al. Non-animal methods to predict skin sensitizarion (I): the cosmetics Europe database. Critical Reviews in Toxicology. 2018;48(5):15.

4. Ryan C, Hulette B, Gerberick G. Approaches for the development of cell-based in vitro methods for contact sensitization. Toxicology in vitro. 2001;15:13.

5. Rustemeyer T, van Hoogstraten IMW, von Blomberg BME, Scheper RJ. Mechanisms in Allergic Contact Dermatitis. In: Rycroft RJG, Menné T, Frosch PJ, Lepoittevin J-P, editors. Textbook of Contact Dermatitis. Berlin, Heidelberg: Springer Berlin Heidelberg; 2001. p. 13-58.

6. Willmet C. Adverse outcome pathways: Development and use in toxicology. Encyclopedia of Toxicology. 2014:95-9.

7. MacKay C, Davies M, Summerfield V, Maxwell G. From pathways to people: Applying the adverse outcome pathway (AOP) for skin sensitisation to risk assessment. Alternatives to animal experimentation. 2013;30(4):473-86.

8. OECD. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins2014.

9. Buehler E. Delayed contact hypersensitivity in the guinea pig. Archives of Dermatology. 1965;91(2):5.

10. Kimber I, Hilton J, Dearman R, Gerberick G, Ryan C, Basketter D, et al. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicology. 1995;103:11.

11. Kimber I, Hilton J, Dearman R, Gerberick F, Ryan C, Basketter D, et al. Assessment of the skin sensitisation potential of topical medicaments using the local lymph node assay: An interlaboratory evaluation. Journal of Toxicology and Environmental Health. 1998;53(7):16.

12. Gerberick G, House R, Fletcher R, Ryan C. Examination of the Local Lymph Node Assay for use in contact sensitization risk assessment. Fundamental and Applied Toxicology. 1992;19:8.

13. OECD. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Organisation for Economic Co-operation and Development. 2010.

14. Gerberick G, Ryan C, Kern P, Schlatter H, Dearman R, Kimber I, et al. Compliation of historical lymph node data for evaluation of skin sensitisation alternative methods. Dermatitis. 2005;16(4):46.

15. Hulette B, Rowden G, Ryan C, Lawson C, Dawes S, Ridder G, et al. Cytokine induction of a human acute myelogenous leukemia cell line (KG-1) to a CD1a+ dendritic cell phenotype. Archives of Dermatological Research. 2001;293:12.

16. Hulette B, Ryan C, Gerberick G. Elucidating changes in surface marker expression of dendritic cells following chemical allergen treatment. Toxicology and Applied Pharmacology. 2002;182:8.

17. Ryan C, Gerberick G, Gildea L, Huletter B, Betts C, Cumberbatch M, et al. Interactions of contact allergens with dendritic cells: Opportunities and challenges for the development of novel approaches to hazard assessment. Toxicological Sciences. 2005;88:8.

18. Ryan C, Gildea L, Hulette B, Dearman R, Kimber I, Gerberick G. Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicology Letters. 2004;150:16.

19. Gildea L, Ryan C, Foertsch L, Kennedy J, Dearman R, Kimber I, et al. Identification gene expression changes induced by chemical allergens in dendritic cells: Opportunities for skin sensitization testing. Journal of Investigative Dermatology. 2006;126:10.

20. Casati S, Aeby P, Basketter D, Cavani A, Gennari A, Gerberick G, et al. Dendritic cells as a tool for the predictive identification of skin sentisization hazard. Alternatives to laboratory animals 2005;33:16.

21. Patlewicz G, Dimitrov S, Low L, Kern P, Dimitrova G, Comber M, et al. TIMES-SS—A promising tool for the assessment of skin sensitization hazard. A characterization with respect to the OECD validation principles for (Q)SARs and an external evaluation for predictivity. Regulatory Toxicology and Pharmacology. 2007;48:15.

22. Dimitrov D, Low L, Patlewicz G, Kern P, Dimitrova G, Comber M, et al. Skin Sensitization: Modeling Based on Skin Metabolism Simulation and Formation of Protein Conjugates. International Journal of Toxicology. 2005;24:16.

23. OECD. Test No. 442C: In Chemico Skin Sensitisation 2020.

24. Gerberick G, Ryan C, Kern P, Dearman R, Kimber I, Patlewicz G, et al. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermatitis 2004;50:15.

25. Gerberick G, Vassallo J, Foertsch L, Price B, Chaney J, Lepoittevin J. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Toxicological Sciences. 2007;97:11.

26. Reisinger K, Hoffmann S, Alepee N, Ashikaga T, Barroso L, Elcombe C, et al. Systemic evaluation of non-animal test methods for skin sensitisation safety assessment. Toxicology in vitro. 2015;29:12.

27. Gerberick F. The use of peptide reactivity assays for skin sensitisation hazard identification and risk assessment. Alternatives to laboratory animals. 2016;44(5):437-42.

28. Gerberick G, Troutman J, Foertsch L, Vassallo J, Quijano M, Dobson R, et al. Investigation or peptide reactivity of pro-hapten skin sensitisers using a peroxidase-peroxide oxidation system. Toxicological Sciences. 2009;112:11.

29. Ryan C, Troutman J, Kern P, Quijano M, Dobson R, Dai H, et al. Refinement of the peroxidase peptide reactivity assay and prediction model for assessing skin sensitisation. Society of toxicology. 2020;178(1):16.

30. Kleinstreuer N, Hoffmann S, Alépée N, Allen D, Ashikaga T, Casey W, et al. Non-animal methods to predict skin sensitization (II): an assessment of defined approaches. Critical Reviews in Toxicology. 2018;48(5):17.

31. Baltazar M, Cable S, Carmichael P, Cubberley R, Cull T, Delagrange M, et al. A Next-Generation Risk Assessment Case Study for Coumarin in Cosmetic Products. Toxicological Sciences. 2020;176(1):236-52.

32. Jaworska J, Dancik Y, Kern P, Gerberick F, Natsch A. Bayesian integrated testing strategy to assess skin sensitisation potency: from theory to practice. Journal of Applied Toxicology. 2013;33:1353-64.

33. Jaworska J, Natsch A, Ryan C, Strickland J, Ashikaga T, Miyazawa M. Bayesian integrated testing strategy (ITS) for skin sensitisation potency assessment: A decision support system for quantitative weight of evidence and adaptive testing strategy. Archives of Toxicology. 2015;89:2355-83.

34. Brownlee J. A gentle introduction to Bayesian belief networks. Machine Learning Mastery. 2019.

35. Jaworska J. Integrated Testing Strategies for Skin Sensitization Hazard and Potency Assessment—State of the Art and Challenges. Cosmetics. 2016;3(16).

REFERENCES

36. Basketter D, Clewell H, Kimber I, Rossi A, Blaauboer B, Burrier R, et al. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing. Alternatives to animal experimentation. 2012;29(1):91.

37. Mahoney C, Ashton R, Birk B, Boobis A, Cull T, Daston G, et al. New ideas for non-animal approaches to predict repeated-dose systemic toxicity: Report from an EPAA Blue Sky Workshop. Regulatory Toxicology and Pharmacology. 2020;104668:8.

38. Piersma A, Burgdorf T, Louekari K, Desprez B, Taalman R, Landsiedel R, et al. Workshop on acceleration of the validation and regulatory acceptance of alternative methods and implementation of testing strategies. Toxicology in Vitro. 2017;50:33.

39. Stuard S, Heinonen T. Relevance and application of read-across - Mini review of European consensus platform for alternatives and Scandinavian Society for Cell Toxicology 2017 workshop session. Basic and clinical pharmacology and toxicology. 2018;123:5.

40. Skare J, Blackburn K, Wu S, Re T, Duche D, Ringeissen S, et al. Use of read-across and computer-based predictive analysis for the safety assessment of PEG cocamines. Regulatory Toxicology and Pharmacology. 2015;71(3):515-28.

41. Wu S, Fisher J, Naciff J, Laufersweiler M, Lester C, Daston G, et al. Framework for identifying chemicals with structural features associated with the potential to act as developmental or reproductive toxicants. Chemical Research in Toxicology. 2013;26:22.

42. Lester C, Reis A, Laufersweiler M, Wu S, Blackburn K. Structure activity relationship (SAR) toxicological assessments: The role of expert judgement. Regulatory Toxicology and Pharmacology. 2018;92:390-406.

43. Abrew AD, Shan Y, Wang X, Krailler J, Kainkaryam R, Lester C, et al. Use of connectivity mapping to support read across: A deeper dive using data from 186 chemicals, 19 cell lines and 2 case studies. Toxicology. 2019;423:11.

44. Agency EC. Read-across assessment framework (RAAF). 2017.

45. Canada H. Use of analogues and read-across in risk assessment 2020 [Available from: https://www.canada.ca/en/health-canada/services/chemical-substances/fact-sheets/analogues-read-across-risk-assessment.html.

46. EPA U. Generalizaed Read-Across (GenRA) Manual [Available from: https://www.epa.gov/chemical-research/generalized-read-across-genra-manual.

47. ChemSafetyPRO. What Is Point of Departure (POD) and How to Use It to Calculate Toxicological Reference Dose (RfD). CSP. 2017.

48. Ellison C, Wu S. Application of structural and functional pharmacokinetic analogs for physiologically based pharmacokinetic model development and evaluation. Regulatory Toxicology and Pharmacology. 2020;114:17.

49. OECD. Guidnace of grouping of chemicals, second edition. Organisation for Economic Co-operation and Development. 2014.

50. Blackburn K, Stuard S. A framework to facillitate consistent characterization of read-across uncertainty. Regulatory Toxicology and Pharmacology. 2014;68:10.

51. ECHA. Read-across illustrative example. European chemicals Agency. 2013.

52. Boyer I, Burnett C, Bergfeld W, Belsito D, Hill R, Klaassen C, et al. Safety Assessment of PEGs Cocamine and Related Ingredients as Used in Cosmetics. International Journal of Toxicology. 2018;37(2):10S-60S.

53. Blackburn K, Carr G, Rose J, Selman B. An interim internal Threshold of Toxicologic Concern (iTTC) for chemicals in consumer products, with support from an automated assessment of ToxCast™ dose response data. Regulatory Toxicology and Pharmacology. 2020(114):1-40.