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EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification

Silvia Casati, Andrew Worth, Patric Amcoff, Maurice Whelan,

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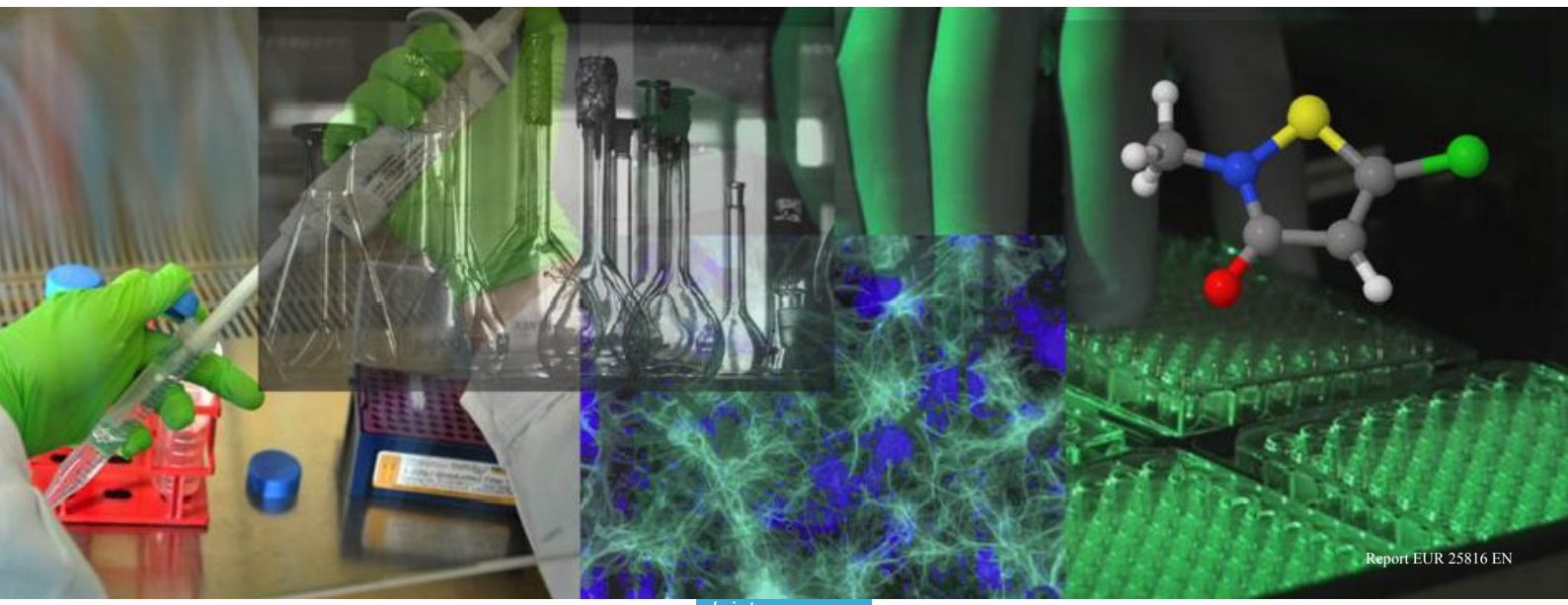

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Abstract

In the absence of validated and regulatory accepted alternative methods, the assessment of the skin sensitisation potential of chemicals still relies on animal testing. Progress in the development of alternative methods has been prompted by the increasing knowledge on the key mechanisms of the skin sensitisation pathway, as recently documented in the OECD Adverse Outcome Pathway for skin sensitisation. Based on an analysis of the regulatory requirements for this endpoint within relevant pieces of EU chemicals legislation, EURL ECVAM has decided to focus its efforts on the development of non-animal testing strategies for skin sensitisation hazard identification and classification, including the subcategorisation of sensitisers, according to the GHS classification system. This would satisfy the majority of the regulatory requirements within the EU and would have a significant impact in terms of replacing animal experiments. This report describes the EURL ECVAM strategy for achieving this goal.

EUROPEAN COMMISSION
Directorate General JRC
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The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification

Content

1. Executive Summary

The identification of chemicals that have the potential to induce skin sensitisation is currently based on the use of animal tests since there are no regulatory accepted alternative (non-animal) methods for this purpose. Despite the mechanistic complexity of the endpoint, important advances in the development of alternative methods have been made in recent times due to the good understanding of the chemistry and biology underlying this toxicological effect, as documented in the Adverse Outcome Pathway (AOP) for skin sensitisation developed by the OECD. This progress is reflected in the numerous submissions of alternative test methods for skin sensitisation that EURL ECVAM has received.

In order to define its own strategy for advancing in the field and for having a framework for the prioritisation of submitted test methods, EURL ECVAM performed an assessment of the regulatory needs for this endpoint within pieces of EU legislation where the generation of skin sensitisation information represents a standard requirement, *i.e.* the Classification Labelling and Packaging of substances and mixtures (CLP) Regulation in its current form and expected revision, the REACH Regulation, the Plant Protection Products (PPP) Regulation, the Biocides Directive and the Cosmetics Directive.

From this analysis it became evident that in order to satisfy all regulatory needs, information on skin sensitisation potency would also be needed. The availability of alternative methods that in isolation or in combination will be able to characterise the potency of a sensitiser would lead to the full replacement of testing on animals for this endpoint. Nevertheless, having non-animal approaches capable of identifying skin sensitisation hazard and generating information that would satisfy classification needs (*i.e.* GHS sub-categorisation) would have the biggest impact on the saving of animals for regulatory testing in the short and medium term.

In the light of this, EURL ECVAM has decided to focus its efforts in the skin sensitisation area for the next five years on the development of non-animal testing strategies suitable for the hazard identification and sub-categorisation of sensitisers. EURL ECVAM will also play a leading role within the OECD to develop a set of complementary Test Guidelines and related guidance documentation that will facilitate a globally accepted approach for skin sensitisation hazard identification and classification.

2. Introduction

Skin sensitisation resulting in Allergic Contact Dermatitis (ACD) is the outcome of a number of complex interactions at molecular, cellular and tissue levels. It is a delayed-type hypersensitivity reaction typically induced by low molecular weight reactive chemicals. It develops in two distinct phases, the induction phase, which sensitises the immune system for an allergic response, and the elicitation phase which occurs following a subsequent contact with the allergen and which leads to the clinical symptoms.

Presently the identification of chemicals that have the potential to induce skin sensitisation is based on the use of animal tests since there are no regulatory accepted alternative methods for this purpose (see Annex). However, despite the complexity of the endpoint, important advances in the development of alternative methods have been made due to the extensive research efforts within both industry and academia. This activity is reflected in the numerous submissions of test methods that EURL ECVAM has received in recent times.

Alternative approaches currently under development and evaluation are designed to address and model the key biological mechanisms of the induction phase of skin sensitisation. These include: 1) the ability of the chemical to penetrate the skin and reach the site of haptenation (skin bioavailability), 2) the covalent binding of the chemical to the skin protein (haptenation), 3) the release of pro-inflammatory signals by epidermal keratinocytes, 4) the activation and maturation of dendritic cells (DC), the skin immuno-competent cells, 5) the migration of DC from skin to the regional lymph nodes and presentation of the antigen to T cells, 6) the proliferation of memory T cells (lymphocytes capable of being stimulated and activated specifically by the haptenated protein) (Adler *et al*. 2011).

Recently the Organisation for Economic Co-operation and Development (OECD) has developed and endorsed an Adverse Outcome Pathway (AOP) specifically for skin sensitisation (OECD 2012a, OECD 2012b). The AOP describes in detail the key biological events or mechanisms underlying skin sensitisation starting from the molecular initiating event (covalent binding of a chemical to skin proteins) through to adverse health effects in humans. Making the collective knowledge on the skin sensitisation process explicit in this way provides an invaluable theoretical and regulatory framework to guide method development, integration and validation.

Moreover, since the AOP comprises a number of key events that must occur before skin sensitisation manifests itself, it is generally accepted that a combination of methods, either *in vitro*, *in chemico* or *in silico*, will be necessary to deliver solutions for achieving full replacement of *in vivo* tests for both hazard identification and risk assessment. However, despite the fact that the AOP and associated key events are well known, it is not yet clear which events or mechanisms are the main determinants of the potency of an allergen. As a consequence, definitive Integrated Testing Strategies (ITS) for potency prediction have yet

to be developed. Considerable progress has been made in the area of hazard identification and thus it is realistic to consider this an achievable goal in the medium term. However, delivering solutions that would satisfy the needs of a full quantitative risk assessment for skin sensitisation, where a more precise prediction of potency is required, remains a challenge.

The purpose of this document is to describe the EURL ECVAM strategy for full replacement of animal testing for skin sensitisation hazard identification and classification (*i.e.* with the ability to discriminate between sensitisers and non-sensitisers and to subcategorise sensitisers according to the two GHS subcategories 1A and 1B). The ultimate aim of the EURL ECVAM strategy is to propose solutions that can satisfy information requirements under EU chemicals legislation, and that can also be considered by the OECD in the context of a globally harmonised approach for skin sensitisation hazard identification and classification.

3. Satisfying Regulatory Needs without the Use of Animals

Considering the information requirements for skin sensitisation within relevant regulations in the EU (see Annex), it is clear that potency information is needed in order to fulfil all the regulatory needs. A good understanding of skin sensitiser potency is particularly important for the safety assessment of cosmetics ingredients where this information is used in combination with information on the expected human exposure to predict the risk for human health and for establishing safe levels of exposure to such ingredients.

The availability of alternative methods that in isolation or in combination will be able to characterise the potency of a sensitiser would lead to the full replacement of animal testing. However, generating hazard information (identification of the sensitisation potential of substances) with non-animal approaches would be sufficient to fulfil the requirements of important pieces of legislation, *i.e.* the Classification Labelling and Packaging of substances and mixtures (CLP) Regulation (EC 2008) in its current form, the REACH Regulation (EC 2006), the Plant Protection Products (PPP) Regulation (EC 2009a) and the Biocides Directive (EC 1998), which would have a considerable impact in terms of animal savings. Moreover, since the risk assessment process requiring potency information is conducted only for those chemicals for which a sensitising hazard has been identified, the availability of alternative methods able to identify non-sensitising chemicals with a sufficient level of accuracy would also contribute to meeting the safety assessment requirements of the Cosmetics Regulation (EC 2009b).

It is foreseen that the 2013 revision of the CLP Regulation will implement the two subcategories 1A “strong sensitisers” and 1B “other skin sensitisers", as adopted in the 3rd revised version of the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) (UN 2011). This in turn may also affect the REACH regulation since

the classification according to the CLP is mandatory for registration of a substance under REACH.

In the light of this, the availability of non-animal methods able to discriminate between sensitisers and non sensitisers and with the capacity to provide insights as to whether or not a sensitiser is a strong sensitiser (sub-category 1A of the GHS) would have the biggest impact in terms of saving of animals for regulatory testing in the short and medium term.

Specifically, in relation to the registration deadlines of the REACH Regulation, the availability of reliable alternative methods able to provide mechanistic insight could already be used in a weight-of-evidence approach to fulfil the information requirements for the 2013 deadline, where assessment of skin sensitisation potential will be needed for approximately 1,000 substances. Regarding the 2018 REACH deadline, ITS for hazard identification, with the ability to assign skin sensitisers to one of the two GHS sub-categories with sufficient accuracy, could potentially fulfil the information requirements for about 10,000 substances. Assuming that at least 20 animals per chemical (3 dose groups, 1 vehicle control group, 1 positive control group, 4 animals/group) would be needed for generating such information with the Local Lymph Node Assay (LLNA) (TG 429) (OECD 2010), which is the preferred test method under REACH, the ability to generate such information with alternative non-animal approaches could lead to the saving of at least 200,000 mice.

The current EURL ECVAM skin sensitisation validation study concerning the assessment of the reliability (*i.e.* transferability, within- and between-laboratory reproducibility) of test methods for skin sensitisation (*i.e.* Direct Peptide Reactivity Assay (DPRA), human-Cell Line Activation Test (h-CLAT) is at an advanced stage, as is the assessment of KeratinoSens, a test methods which underwent external (non-ECVAM coordinated) validation. So far it appears that a number of the methods (*i.e.* DPRA, KeratinoSens and h-CLAT) are adequately reproducible to be considered for inclusion in an ITS for hazard identification. In addition, there are already published studies describing how these methods can be employed in combination to increase the accuracy of prediction of skin sensitisation hazard when compared to the performance of individual methods (Natsch *et al.,* 2009; Jaworska *et al.,* 2011*;* Bauch *et al*., 2012; Nukada *et al*., 2012).

Using the results of the ECVAM validation study, consideration is also being given to how these methods can contribute to decisions regarding sub-categorisation of skin sensitising chemicals (*i.e.* sub-category 1A and sub-category 1B of the GHS). A complementary project is being pursued by the Cosmetics Europe Skin Tolerance Task Force to evaluate how a larger set of *in vitro* test methods for skin sensitisation can contribute to the generation of potency information.

The OECD has already expressed interest in *in vitro* methods for skin sensitisation by including some of them (*i.e.* DPRA and KeratinoSens) in its 2012 work program. In the third quarter of 2012 the European Commission/EURL ECVAM jointly with the Japanese Center for the Validation of Alternative Methods (JaCVAM) submitted a Standard Project

Submission Form (SPSF) to the OECD Test Guidelines Programme for the development of a Test Guideline for the h-CLAT.

4. EURL ECVAM Strategy

EURL ECVAM will focus its efforts on developing one or more ITS for the full replacement of animal testing for skin sensitisation hazard identification and classification (*i.e.* with the ability to discriminate between sensitisers and non-sensitisers and to subcategorise sensitisers according to the GHS). The intention is to demonstrate that these ITS are adequate to satisfy the information requirements of EU chemicals legislation, in particular CLP (*i.e.* GHS classification and subcategorisation), REACH, PPP and Biocides. In addition, the European Commission/EURL ECVAM will take a leading role within the OECD, the ultimate aim of which is to develop a set of complementary Test Guidelines and related Guidance Documents that will facilitate a globally accepted approach for skin sensitisation hazard identification. To achieve this, EURL ECVAM will pursue the following short, medium and long term goals (Figure 1).

4.1. Short term goals (2013-2014)

To exploit the methods currently undergoing formal evaluation, EURL ECVAM will:

- Finalise the validation and ECVAM Scientific Advisory Committee (ESAC) peer review of the test methods currently under evaluation (DPRA, h-CLAT, and KeratinoSens) and, contingent upon the outcome of the peer-review, support the OECD activities for the development of Test Guidelines for these methods.
- Take leadership at the OECD for the development of AOP-based ITS/Integrated Approaches to Testing and Assessment (IATA) for skin sensitisation. It is foreseen that this programme will be under the responsibility of the Hazard Assessment Task Force (HATF) and will be carried out in close cooperation with the Extended Advisory Group (EAG) on Molecular Screening and Toxicogenomics.
- Explore the user requirements for an EURL ECVAM database on skin sensitisation, to be developed in cooperation with various collaboration partners and stakeholders. This would include data generated by the methods undergoing formal evaluation by EURL ECVAM but would not be limited to these. Further population of the database may be based on a public call for *in vitro/in vivo* data.
- Initiate an in-house project aimed at the evaluation of method combinations (*in silico, in chemico* and *in vitro*) for hazard identification and classification. It is anticipated that as final outcome of this project multiple ITS will be developed, based on different user requirements. The development of ITS will take into consideration method combinations already proposed and in use by some companies.
- Start discussions about the process for implementing future ITS in the EU legislation and at OECD level.

4.2. Medium term goals (2014-2015)

The medium term goals, focusing on the improvement of a preliminary ITS approach, are to:

- Identify gaps and make recommendations for further studies to generate relevant data to improve the predictive capacity and expand the applicability of ITS.
- Evaluate the need to include in the ITS information on epidermal disposition and dermal metabolism (including, for example, information generated by QSARs, artificial membrane barriers, and *in vitro* methods).
- Investigate the potential of mechanistic QSARs for predicting key events along the skin sensitisation pathway, with a view to integrating such models in the ITS.

4.3. Long term goals (2016 and beyond)

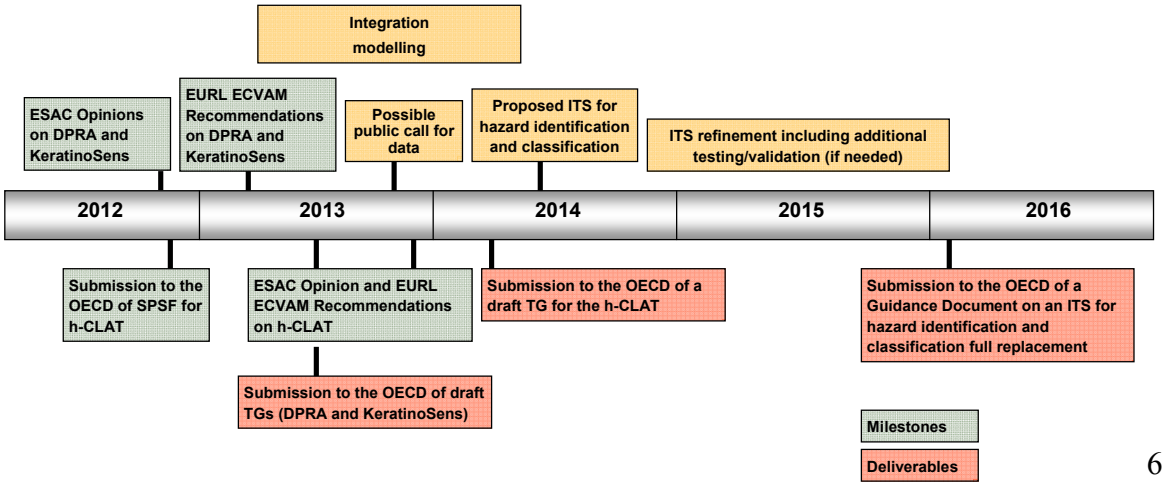
The long term goals, focusing on regulatory acceptance and implementation, are to:

- Propose scientific solutions (ITS) based on combinations of alternative methods for the prediction of skin sensitisation potential and for classification purposes, with a view to fully satisfying regulatory information requirements in the EU (*e.g.* CLP and REACH).

- Take a leading role in OECD activities on the development of Test Guidelines and guidance documentation, in order to facilitate a globally harmonised approach to the identification and classification of chemicals based on skin sensitisation potential.

- Collaborate with partners working towards scientific solutions (ITS) for potency assessment, to facilitate quantitative safety assessments for the skin sensitisation endpoint.

Figure 1: EURL ECVAM roadmap for achieving skin sensitisation hazard identification and classification based on alternative methods.



5. Evaluation of Test Methods Submitted to EURL ECVAM

EURL ECVAM will continue evaluating submitted test methods in the light of its overall strategy. Therefore the assessment of incoming submissions will be primarily based on the value of information derived from these methods with respect to the methods currently under evaluation. In addition, new methods that provide equivalent information will also be considered in terms of possible advantages concerning ease of use, cost, throughput and widespread availability. In order to facilitate the comparison and evaluation of submitted test methods, EURL ECVAM may request test submitters to provide data on a specific set of reference chemicals (*e.g.* including those from the current EURL ECVAM validation study). Such data will likely be included in the EURL ECVAM skin sensitisation database serving as input to ITS development.

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Annex. Regulatory Requirements for Skin Sensitisation

1. Regulatory Requirements

Information on the skin sensitisation potential of substances represents an important requirement in the context of several pieces of EU legislations aiming at the protection of human health and the environment as summarised in Table 1. The following section illustrates the information requirements for skin sensitisation within the regulations considered for the purpose of this report.

1.1. Classification Labelling and Packaging

The ***CLP Regulation (EC) No 1272/2008 for "Classification, Labelling and Packaging of substances and mixtures"*** (EC 2008) ensures that the hazards presented by chemicals are clearly communicated to workers and consumers in the European Union through appropriate hazard symbols (pictograms) and labelling phrases. The CLP Regulation entered into force in January 2009, and the method of classifying and labelling chemicals is based on the United Nations' Globally Harmonised System (GHS) (UN 2011). The CLP Regulation replaces two pieces of EU legislation, the Dangerous Substances Directive and the Dangerous Preparations Directive and for this a transition period until 2015 is foreseen.

Currently, the CLP Regulation allows classification of skin sensitisers in one hazard category (Category 1) on the basis of existing evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons or if there are positive results from an appropriate animal test. Since it is possible to refine the evaluation of the skin sensitisers on the basis of the potency of the sensitising effect, guidance is provided on how to evaluate the potency on the basis of the recommended test methods. However, classification of sensitisers into potency categories is currently not a requirement in the CLP, rather it is used to set specific concentration limits for chemicals, classified as skin sensitisers constitued in mixtures. Classification based on potency consideration is expected to be introduced in a future amendment of CLP (most probably already in 2013) for complete implementation of GHS.

Within the GHS, skin sensitisers can be assigned to subcategory 1A "strong sensitisers" or to subcategory 1B "other skin sensitisers" using a weight of evidence approach on the basis of reliable and good quality evidence from human cases or epidemiological studies and/or observations from appropriate studies in recognised and officially accepted animal tests.

1.2. Chemicals subject to REACH

The CLP Regulation and the ***REACH Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals*** (EC 2006a) are closely interlinked since the classification of a substance is a mandatory part of the REACH registration process.

REACH applies to all chemical substances, both those used in industrial processes and those contained in consumer products. To comply with the Regulation, companies must identify and communicate the risk management measures to the users for the substances they manufacture and market in the EU.

The REACH Regulation specifies the information that shall be submitted to ECHA for registration and evaluation purposes. The assessment of the skin sensitisation potential of substances is among the standard information requirements for substances manufactured or imported in the EU in quantities of one tonne or more. (Annex VII of the REACH Regulation). Therefore, information on skin sensitisation is a mandatory requirement for substances produced or imported with the lowest tonnage level, affecting all chemicals registered under REACH. The assessment of this endpoint comprises two consecutive steps: firstly an assessment of the available human, animal and alternative data and secondly *in vivo* testing.

Animal testing does not need to be conducted in case there is available information to classify the substance for skin sensitisation or corrosivity, in case the substance is a strong acid or base, or in case the substance is flammable at room temperature. The Local Lymph Node Assay (LLNA: OECD 2010a) is the first choice method for *in vivo* testing. The use of other *in vivo* methods is accepted, although their use shall be scientifically justified.

The precondition within REACH is that before any new *in vivo* tests are carried out to fulfil the information requirements, all available *in vitro* data, *in vivo* data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across or categories) shall be assessed first, as described in the general rules for adaptation of the standard testing regime (Annex XI of the REACH Regulation).

Under REACH, manufacturers, importers and downstream users should ensure that they manufacture, place on the market or use substances in such a way that they do not adversely affect human health. REACH Annex I describes how manufacturers and importers have to assess and document that the risks arising from the substance they manufacture or import are controlled during manufacture and their own use(s) and that others further down the supply chain can control the risks. REACH (Annex I, 1.0.1) defines the Derived No-Effect Level (DNEL), *i.e.* the level of exposure above which humans should not be exposed. In the risk characterisation, the exposure of each human population known to be or likely to be exposed is compared with the appropriate DNEL. The risk to humans can be considered to be controlled if the exposure levels estimated do not exceed the appropriate DNEL.

Skin sensitisation is generally regarded as a threshold effect, although in practice it may be very difficult to derive a threshold value and to set a DNEL. Normally only a qualitative assessment can be performed, however, the human data and data derived from the LLNA may be in some cases used in a more quantitative manner.

Thus, the general approach to sensitizers could be viewed as a two-step procedure involving:

a) A qualitative approach (through potency categorization) to define the Risk Management Measures (RMMs) and Operational Conditions (OCs).

b) Where possible, by derivation of a DNEL to judge the remaining/residual likelihood of risks after these RMMs and OCs are implemented.

RMMs and OCs may be chosen in relation to the potency of the sensitizer. The more potent sensitizer, the more stringent measures to control exposure are required.

To provide practical guidance for the qualitative approach a hierarchy/categories of hazard (high, moderate and low) is proposed (ECHA, 2008). In cases where the available data does not allow potency categorisation of a sensitising substance, the RMMs and OCs applicable to the highest hazard category should be summarised.

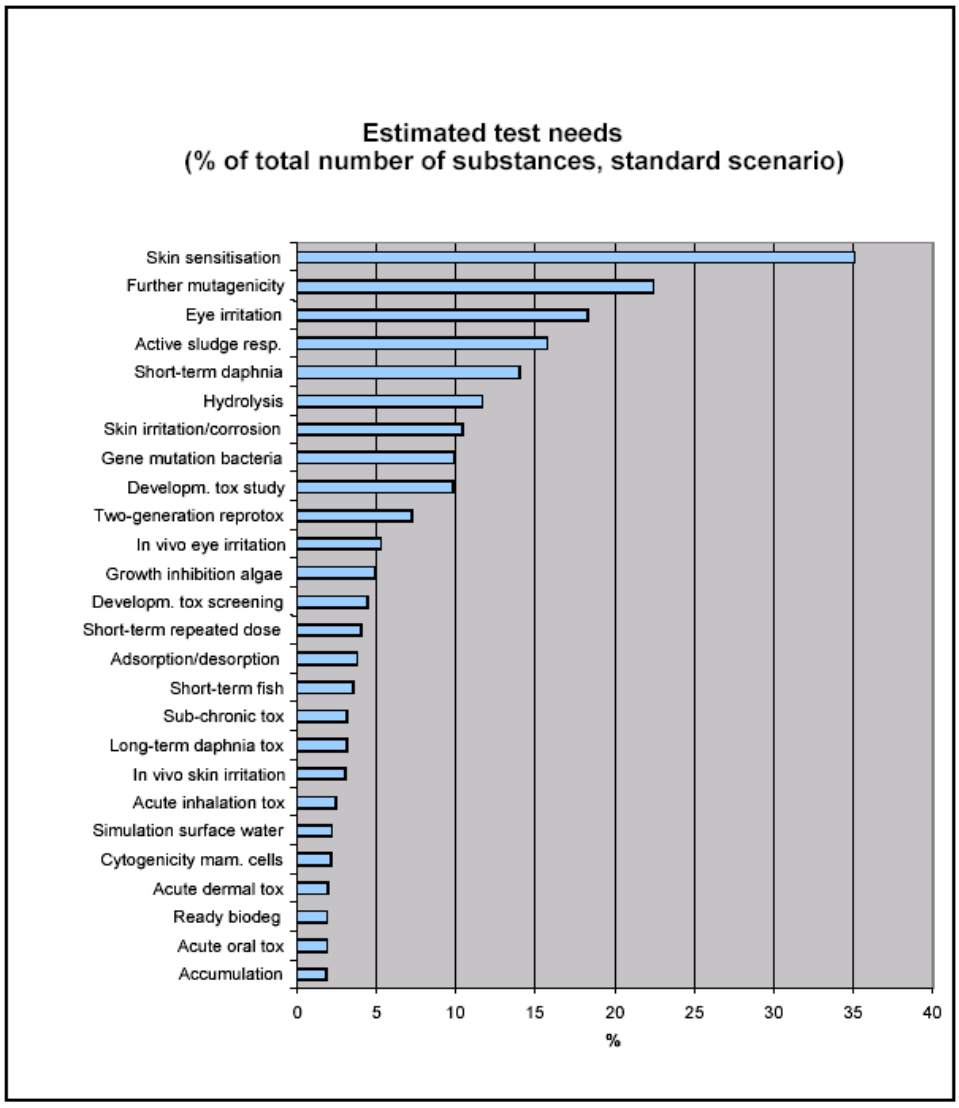
In summary, skin sensitisation potency information does not represent a legal requirement within REACH, however, if such information can be obtained from the data available it can be used to refine the risk assessment performed by manufactures/importers.

REACH requires all companies manufacturing or placing a substance on the EU market in quantities greater than one tonne per year to register that substance with ECHA. The deadline of REACH registration depends on the tonnage band of a substance. The 31 May 2013 is the deadline for industry to register all phase-in substances manufactured or imported in the EU at or above 100 tonnes per year. The number of phase-in substances (produced or imported with volumes of over 100 tonnes per year) intended to be registered by 31 May 2013 is assumed to be approximately 3551 (ECHA website http://echa.europa.eu/web/guest/reach-2013). From this number one can exclude 866 substances already registered for the 2010 deadline (>1,000 tonnes per year) leaving 2685 substances still to be registered by 2013. According to a report prepared by the former European Chemicals Bureau it was estimated that skin sensitisation test is the test that needs to be conducted for the highest percentage (35%) of the phase-in substances (Figure 2). On the basis of this assumption, about 1,000 of the 2,685 substances will require information on their skin sensitisation potential.

Phase-in substances manufactured or imported in volumes of over 1 tonne per year will need to be registered by the 31st May 2018. It is estimated that for approximately 20,000 chemicals skin sensitisation information will be required. Assuming that grouping/read-across may reduce this testing requirement by up to 50%, still about 10,000 chemicals will

have to be tested for skin sensitisation using animal tests unless alternative test methods becomes available in the meantime.

Figure 2: Estimated percentage of the total number of phase-in substances that will need to be tested for the different endpoints under REACH (Van der Jagt *et al.* 2004).



1.3. Plant Protection Products

Regulation (EC) No 1107/2009 concerning the placing of plant protection products (PPP) on the market (EC 2009a) replaces Council Directive 91/414/EEC. Under the new PPP regulation data requirement for active substances and pesticide products are laid down in two separate regulations namely Regulation (EU) No 544/2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for active substances (EU 2011a) and PPP Regulation (EC) No 545/2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for plant protection products (PPP) (EU 2011b).

In the context of the PPP Regulation information on the skin sensitisation potential is mandatory for both the active substances contained in plant protection product and the final product. Testing for skin sensitisation of both the active substance(s) and the final product does not need to be conducted if the active substance or co-formulants are known to have sensitising properties. If sensitisation information from a valid test is not provided the dossier is incomplete. Skin sensitisation potency information does not constitute a regulatory requirement for this piece of legislation since the endpoint is not considered for risk assessment purposes (EC 2006b).

1.4. Biocides

Directive 98/8/EC concerning the placing of biocidal products on the market (EC 1998**)** regulates the placing of biocidal products (any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action) on the EU market. It sets out a Community harmonised system for the authorisation and placing on the market of biocidal products; for the mutual recognition of these authorisations within the Community; and for the establishment at Community level of a positive list of active substances which may be used in biocidal products. It aims to ensure a high level of protection for human and animal health and for the environment. Directive 98/8/EC will be replaced by Regulation (EU) No 528/2012 (EU 2012) concerning the making available on the market and use of biocidal products, which will enter into force on 1 September 2013. A technical guidance document in support of the implementation of the new directive is currently under preparation.

According to the "Technical Guidance Document in Support of the Directive 98/8/EC concerning the placing of biocidal products on the market" and laying down the data requirements for active substances and biocidal product, skin sensitisation hazard information belong to the core data set being, therefore, mandatory for both the active substance and the final product. As for the PPP Regulation, potency information does not constitute a regulatory requirement. According to the current draft of the technical guidance document being prepared in support of the new regulation probably no substantial changes will be made to the information requirements for skin sensitisation.

1.5. Cosmetics

Article 2 of ***Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products*** (EU 1976) specifies that a cosmetic product must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use. The responsibility to demonstrate that the product is safe under the expected exposure conditions lies with the cosmetic manufacturer or his authorized agent or by any other person responsible for placing the product on the Community market.

Directive 76/768/EEC will be replaced by a new Regulation (EC) No.1223/2009 (EC 2009b) on cosmetic products which is not fully applicable until 11 July 2013.

Annex I of Regulation (EC) No.1223/2009 specifies the minimum information which needs to be reported in the Cosmetic Product Safety Report. In relation to the requirements on the toxicological profile of the substances, Annex I explicitly mentions skin sensitisation as one of the toxicological endpoint necessitating particular focus together with local toxicity evaluation (skin and eye irritation) and photo-induced toxicity in case of UV absorption. More guidance is provided by the Scientific Committee on Consumer Safety (SCCS) in the "*SCCS's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, 7th Revision*" (EC 2010). The document provides guidance to public authorities and cosmetic industry, in order to improve harmonised compliance with Directive 76/768/EEC and in particular with the sixth (EU 1993) and the seventh (EC 2003) amendments of this Directive. It also sets out the essential information required for safety files submitted for evaluation to the SCCS. According to the same guidance the risk assessment process for cosmetics ingredients should be conducted according to the following four steps:

1) **Hazard identification** based on the results of *in vivo* tests, *in vitro* tests, clinical studies, accidents, human epidemiological studies and, when available, quantitative structure activity relationship (QSAR) studies. The intrinsic physical, chemical and toxicological properties of the molecule under consideration are studied to identify whether the substance has the potential to damage human health.

2) **Dose-response assessment** in which the relationship between the toxic response and the exposure is studied. In the case of an effect with a threshold, the dosage at which No Adverse Effect Levels are observed (NOAEL), is determined. If the NOAEL is not available, the lowest dosage at which an adverse effect is observed (LOAEL) is used.

3) **Exposure assessment** in which the amount and the frequency of human exposure to the compound are determined (including potential specific groups at risk, *e.g.* children, pregnant women, etc.).

4) **Risk characterisation:** the probability that the substance under investigation causes damage to human health and the level of risk, are examined.

Risk assessment for skin sensitisation relies on the above elements. The approaches currently used rely on a good understanding of the potency of the sensitiser. Such information is considered in the context of the ingredient's concentration in the product and the predicted human exposure scenario.

The Cosmetics Directive and its 7th amendment introduce an end to animal testing by imposing bans on:

- testing finished cosmetic products and ingredients on animals (**testing ban**);
- marketing finished cosmetic products which have been tested on animals or which contain ingredients that have been tested on animals (**marketing ban**).

The ban of animal testing of finished cosmetics product has been in force since September 2004, whereas the animal testing ban of ingredients and combination of ingredients entered into force in March 2009. With regard to the marketing ban, this applies since March 2009 to all human health endpoints. In relation to tests concerning skin sensitisation, carcinogenicity, reproductive toxicity, repeated-dose toxicity and toxicokinetics, marketing ban will apply from March 2013 regardless of the availability of alternative test methods.

As part of the implementation of the 2013 marketing ban, in 2011 the Commission had to report to the European Parliament and the Council in case alternatives to animal testing for the above mentioned human health effects will not be developed and validated by the 2013 deadline. In that case the Commission shall elaborate a legislative proposal.

Against this background, in 2010 the Commission invited scientific experts proposed by various stakeholders to review the state-of-play of alternative methods and to provide a science-based estimate of the time necessary to achieve full replacement of animal testing for the complex endpoints (Adler *et al.* 2011). For the area of skin sensitisation it was estimated that by 2017-2019 full replacement of animal tests might be achieved but that methods able to discriminate between sensitisers and non-sensitisers might become available earlier.

Skin sensitisation represents an important endpoint for the consumer safety because of the high frequency of allergic reactions among the undesirable effects of cosmetic products. Accordingly, skin sensitisation represents the highest testing data need for the cosmetics sector with an average of 151 animal tests for skin sensitisation conducted per year by large companies and representing about 71% of the number of animal tests conducted per year for the 2013 endpoints (EC, 2011).

It is important to note that 90% of ingredients used in cosmetics are being used in other areas (*e.g.* REACH, CLP, Biocidal Products etc.; (EC, 2011)).

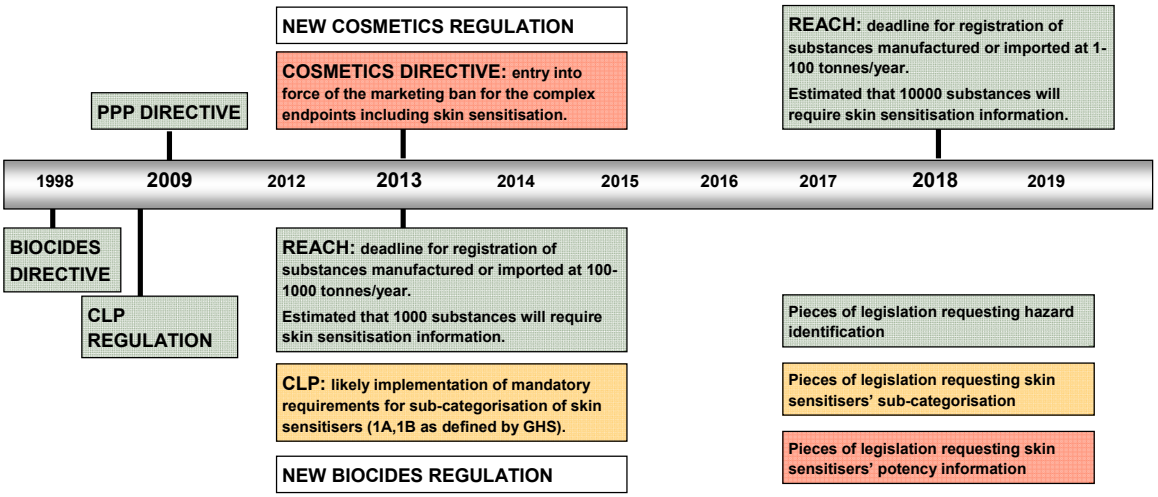
The information requirements described above for the five different pieces of legislations considered for the purpose of this report are summarised in Table 1. The same requirements are highlighted in Figure 3 concerning the timeline for the implementation of the different regulations.

Table 1: Overview of the EU legislations considered in the context of this report and their information requirements for skin sensitisation.

EU Regulation/Directive	Application	Information requirements	Method of choice
CLP Regulation (EC) No 1272/2008 (for "Classification, Labelling and Packaging of substances and mixture" amending and repealing Directive 67/548/EEC (Dangerous Substances Directive) and 1999/45/EC (Dangerous Preparations Directive). CLP is based on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) and is implementing the provisions of the GHS within the EU	All substances and mixtures placed on the market irrespective of the tonnage level, fall within the scope of classification under CLP and should be evaluated in order to reach a decision as to whether they should be classified or not. All substances subject to REACH are also subject to classification, even those not placed on the market if they are subject to registration or notification.	**Hazard assessment and appropriate labelling** **Currently allows classification into one hazard category**. **Classification into potency categories** is currently not a requirement in the classification of sensitisers even though **expected to be introduced in a future amendment of CLP** for complete implementation of GHS. Meanwhile for the application of the CLP criteria guidance is provided for refining the evaluation of skin sensitisers on the basis of their potency in the case this information is available.	For new testing of substances the LLNA (TG 429) is now the method of first choice. In the exceptional circumstance that the LLNA is not appropriate, one of the alternative tests may be used (Buehler or guinea-pig Maximisation test (GPMT) (TG 406), but justification shall be provided.
REACH Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals.	Skin sensitisation testing is mandatory for all substances manufactured or imported in the EU in quantities of one ton or more per year (lowest tonnage level) Considered to be the endpoint with the highest testing needs for phase-in substances.	**Hazard assessment** Quantitative and/or qualitative risk characterisation is performed when potency information is available (from existing data or newly generated data) to refine the risk management measures and operational conditions and to derive a no-effect level (DNEL) to judge the residual likelihood of risk once such measures are implemented.	For new testing of substances the LLNA (TG 429) is the method of first choice. In the exceptional circumstance that the LLNA is not appropriate, one of the alternative tests may be used (Buehler or GPMT), but justification shall be provided.
PPP Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market Regulation (EU) No 544/2011implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for active substances PPP Regulation (EU) No 545/2011 implementing Regulation (EC) No	All plant protection products and active substances marketed in the EU independent of tonnage level Information requirement for skin sensitisation mandatory for both active substances contained in PPPs and the final product.	**Hazard assessment** The test is not needed if the active substance is classified as a sensitiser according to Directive 67/548/EEC or is otherwise known to be a sensitiser (from human data).	The GPMT (TG 406) is considered to be the preferred adjuvant technique in certain cases there may be good reasons for choosing the Buehler test (TG 406) or the LLNA (TG 429). However, scientific justification may be given when either of the two latter tests is used.

1107/2009 of the European Parliament and of the Council as regards the data requirements for plant protection products (PPPs)			
Biocidals Directive (98/8/EC) concerning the placing of biocidal products on the market	All biocidal products and active substances marketed in the EU independent of tonnage level Information requirement for skin sensitisation mandatory for both active substances contained in biocidal products and the final product.	**Hazard assessment** The test is not needed if the active substance is classified as a sensitiser according to Directive 67/548/EEC or is otherwise known to be a sensitiser (from human data).	The GPMT (TG 406) is considered to be the preferred adjuvant technique in certain cases there may be good reasons for choosing the Buehler test (TG 406) or the LLNA (TG 429). However, scientific justification may be given when either of the two latter tests is used.
Cosmetics Directive (76/768/EEC) and 7th Amendment (Directive 2003/15/EC)	All cosmetics ingredients independent of tonnage level. Skin senstisation is among the most relevant endpoints for the toxicological profile of cosmetics ingredient (considered to be the endpoint with the highest testing needs for the cosmetic sector) Information requirement for skin sensitisation included in the minimal base set requirement for inclusion of a substance in one of the Annexes to Directive 76/768/EEC.	**Hazard assessment + Quantitative risk characterisation**	All regulatory accepted test methods for skin sensitisation (LLNA, TG 429; GPMT/BT TG 406). In practice the test of choice is the LLNA since it provides potency information. The ban on animal testing entered into force in 2009 however until 2013 industries will be allowed to place on the marked cosmetics containing ingredients tested on animals if testing was performed outside Europe. This will be no longer possible as from 2013 when the marketing ban on cosmetics containing ingredients tested on animals will entry into force

Figure 3: Timeline for implementation of EU legislation and skin sensitisation information requirements.



2. Regulatory Tests

In the absence of validated alternative methods, regulatory accepted tests suitable to identify and characterise substances causing allergic contact dermatitis in humans rely on the use of animals. These include: the traditional guinea pig tests (Buehler Test and Guinea-pig Maximisation Test; OECD TG 406 (OECD 1992)) and the Local Lymph Node Assay (LLNA: OECD TG 429 (OECD 2010a)) including its non-radioactive variants (Local Lymph Node Assay: DA, OECD TG 442A (OECD 2010b) and Local Lymph Node Assay: BrdU Elisa, OECD TG 442B OECD (2010c)). The LLNA is considered a reduction/refinement method with respect to the traditional guinea-pig tests since it generally involves the use of fewer animals and entails less stressful procedures. The guinea-pig tests comprise both the induction phase and the elicitation phase of the immune response whereas the LLNA covers the mechanisms underlying the induction phase only. An overview of the regulatory accepted animal tests is provided in Table 2.

Table 1: Overview of the regulatory accepted animal tests for skin sensitisation

OECD Test Guidelines for Skin Sensitisation	Skin sensitisation phases covered	Animal species	Adjuvant	Exposure	Dose levels	N° of animals in control/ treatment group	Number of animals per test according to TG	Test duration (days)	Endpoint	Classification criteria
406: Guinea Pig Maximisation Test (GPMT)	Induction + elicitation	Guinea pig	Yes (Freund's Complete Adjuvant-FCA)	*Induction*: intradermal injections (day 0) and topical application (day 5-7 and day 6-8) *Challenge:* topical application (day 20-22) *by occluded patch* *Re-challenge:* possible	*Induction*: 1 dose (highest concentration to cause mild-to moderate-skin irritation) *Challenge:* 1 dose (highest non-irritant dose)	10/20	30	23-25	Skin reactions (erythema/ oedema)	Positive reaction in at least 30% of the animals in the treatment group
406: Buehler Test	Induction + elicitation	Guinea pig	No	*Induction*: topical application (day 0, day 6-8 and day 13-15) *Challenge:* topical application (day 27-29) *Re-challenge:* possible	*Induction*: 1 dose (highest concentration to cause mild skin irritation) *Challenge:* 1 dose (highest non-irritant dose)	10/20	30	30-32	Skin reactions (erythema/ oedema)	Positive reaction in at least 15% of the animals in the treatment group
429: Local Lymph Node Assay	Induction	Mouse	No	Topical application	At least 3 doses (highest dose should not give systemic toxicity and/or excessive local irritation)	4/4	20	6	Cellular proliferation in auricular lymph nodes measured by radioactive labeling	Stimulation Index (SI) >3 at any dose.
442A: Local Lymph Node Assay: DA	Induction	Mouse	No Pre-treatment with 1% Sodium Lauryl Sulphate (SLS)	Topical application	At least 3 doses (highest dose should not give systemic toxicity and/or excessive local irritation)	4/4	20	8	Cellular proliferation in auricular lymph nodes quantified by determination of ATP content	Stimulation Index (SI) ≥1.8 at any dose.
442B: Local Lymph Node Assay: BrdU Elisa	Induction	Mouse	No	Topical application	At least 3 doses (highest dose should not give systemic toxicity and/or excessive local irritation)	4/4	20	6	Cellular proliferation in auricular lymph nodes quantified by determination of BrdU incorporation	Stimulation Index (SI) ≥1.6 at any dose.

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