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EURL ECVAM Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing

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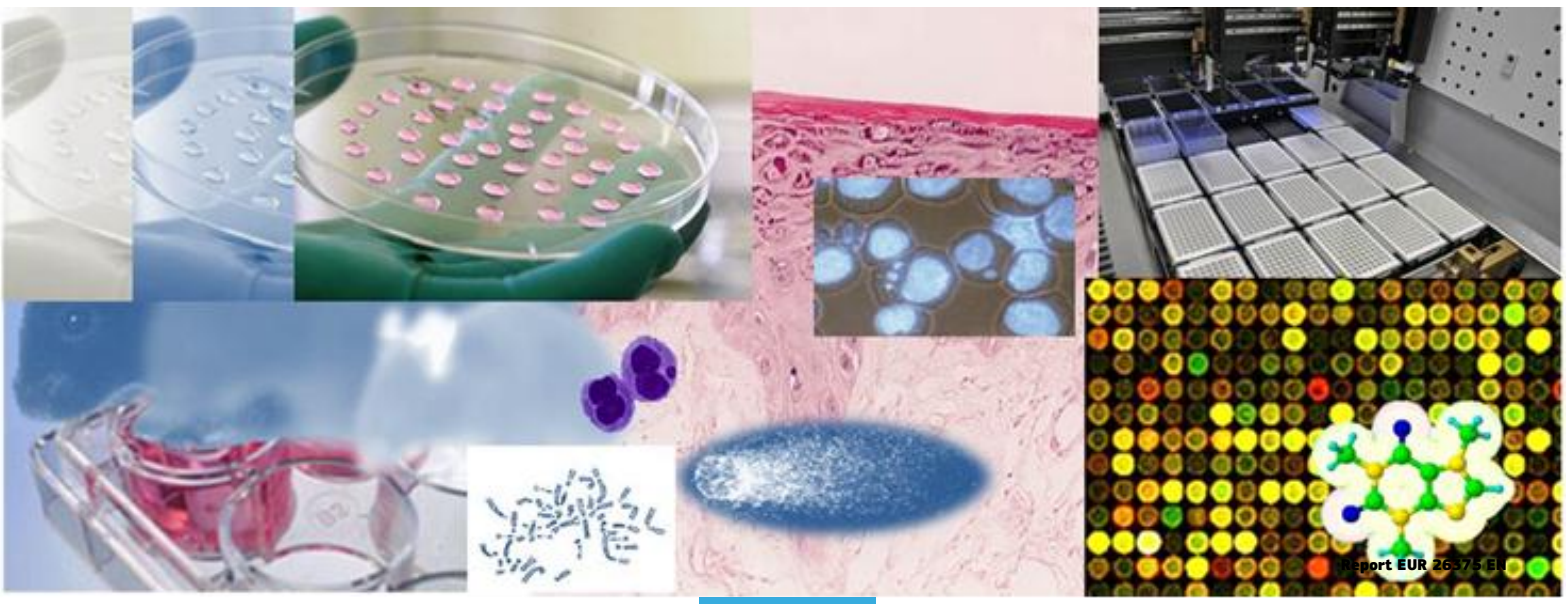
2013



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This publication is a Reference Report by the Joint Research Centre of the European Commission.

JRC86616

EUR 26375 EN

ISBN 978-92-79-34844-0 (pdf)
ISBN 978-92-79-34845-7 (print)

ISSN 1018-5593 (print)
ISSN 1831-9424 (online)

doi: 10.2788/43865 (online)

Luxembourg: Publications Office of the European Union, 2013

Printed in Luxembourg

EUROPEAN COMMISSION
Directorate General JRC
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection (Ispra)
The EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing

Executive Summary

The assessment of genotoxicity represents an important component of the safety assessment of all types of substances, relevant in the context of EU and international legislation aimed at the protection of human health. In general, the assessment of genotoxic hazard to humans follows a step-wise approach, beginning with a basic battery of *in vitro* tests followed in some cases by *in vivo* testing. Regulatory requirements, in particular for *in vivo* testing, vary depending on the type of substance under regulation and the region.

In order to develop its own strategic plan for advancing the field and for having a framework for the prioritisation of *in vitro* test methods submitted for evaluation, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) performed an assessment of the regulatory needs for this endpoint within pieces of EU legislation where the generation of genotoxicity information is a standard requirement. The EURL ECVAM strategy is based on the state of the science in the field of genotoxicity, including recent and on-going efforts. Although several *in vitro* tests are available at different stages of development and acceptance, they cannot at present be considered to fully replace animal tests currently used to evaluate the safety of substances. In light of this, EURL ECVAM proposes a pragmatic approach to improve the traditional genotoxicity testing paradigm that offers solutions in the short-term and medium-term and that draws on the considerable experience of 40 years of regulatory toxicology testing in this area. In the longer term, progress in mechanistic understanding and biomedical technologies will likely provide opportunities to consider completely new approaches and assessment strategies. However this will depend heavily on continued investment in research and development and the feasibility of translating research results into robust solutions fit for regulatory use.

EURL ECVAM considers therefore that efforts should be directed towards the overall improvement of the current testing strategy for genotoxicity, which provides a means of avoiding or minimising the use of animals, according to the regulatory context. Several opportunities for improving the testing strategy have been identified which aim to: i) enhance the performance of the *in vitro* testing battery so that fewer *in vivo* follow-up tests are necessary; and ii) guide more intelligent *in vivo* follow-up testing to reduce and optimise the use of animals. The implementation of this strategic plan will rely on the cooperation of EURL ECVAM with other existing initiatives and coordinated contributions from various stakeholders.

Abbreviations and Glossary of Terms

3Rs	Refinement, Reduction, Replacement
BMBF	Federal Ministry for Education and Research (Germany)
CGX	The Carcinogenicity and Genotoxicity eXperience (CGX) database
CLP	Classification, Labelling and Packaging of Substances and Mixtures
COM	UK Committee on Mutagenicity of Chemicals in Food, Consumers Products and the Environment
CTA	Cell Transformation Assay
EC	European Commission
EC No.	EC number, refers either to EINECS number, ELINCS number, NLP number or, EC Number appointed under REACH procedure
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EU	European Union
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
GHS	Globally Harmonised System of classification and labelling of chemicals
HET-MN	Hen's Egg Test- Micronucleus
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ILSI/HESI	International Life Sciences Institute/Health and Environmental Sciences Institute
ITS	Integrated Testing Strategy
JRC	Joint Research Centre (of the European Commission)
MLA	Mouse Lymphoma Assay
NC3Rs	National (UK) Centre for Refinement, Reduction, Replacement
NIHS	National Institute of Health Sciences, Japan
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
SCCP	Scientific Committee on Consumer Products (European Commission)
SCCS	Scientific Committee on Consumer Safety (European Commission)
SHE	Syrian hamster embryonic stem cells
TG	Test Guideline
UDS	Unscheduled DNA Synthesis
US FDA	US Federal Drug Administration
US NTP	US National Toxicology Program
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

Contents

1. Introduction

Genetic alterations in somatic and germ cells are associated with serious health effects, which in principle may occur even at low exposure levels. Mutations in somatic cells may cause cancer if they occur in proto-oncogenes, tumour suppressor genes and/or DNA damage response genes (Erickson, 2010). Accumulation of DNA damage in somatic cells has also been proposed to play a role in degenerative conditions such as accelerated ageing, immune dysfunction, and cardiovascular and neurodegenerative diseases (Hoeijmakers, 2009; Slatter and Gennery, 2010; De Flora and Izzotti, 2007; Frank, 2010). Mutations in germ cells can lead to spontaneous abortions, infertility or heritable damage to the offspring and possibly to subsequent generations.

The assessment of genotoxicity represents an important component of the safety assessment of all types of substances (e.g. pharmaceuticals, industrial chemicals, pesticides, biocides, food additives, cosmetic ingredients) relevant in the context of EU and international legislation aimed at the protection of human health (ICH, 2011; VICH, 2012; EC no. 1907/2006; EC nos. 1107/2009b and 283/284-2013; EC no. 528/2012; EFSA, 2011; EC no 1223/2009 and SCCS'S notes of guidance, 1501/2012). The results of genotoxicity tests inform the cancer risk assessment process and are used for classification and labelling (C&L) of chemicals in the EU (the REACH regulation No 1907/2006 and Regulation (EC) No. 1272/2008 on the classification, labelling and packaging (CLP) of substances and mixtures) and across the world (UN Globally Harmonised System, GHS, 2011).

Genotoxicity testing includes the measurement of DNA primary damage that can be repaired and is therefore reversible, as well as the detection of stable and irreversible damage (i.e. gene mutations and chromosome aberrations) that are transmissible to the next generation when they occur in germ cells, and the perturbation in mechanisms involved in the preservation of the integrity of the genome. For an adequate evaluation of the genotoxic potential of a chemical substance, different endpoints (i.e. induction of gene mutation, structural and numerical chromosome alterations) have to be assessed, as each of these events has been implicated in carcinogenesis and heritable diseases. These changes may involve a single or block of base pairs, single gene or gene segment, or a block of genes or chromosomes. The term mutagenicity refers to changes in nucleotide sequence often referred to as point mutations that involve a small number of nucleotides such as base pair substitutions, small insertions and deletions, while the term clastogenicity is used for agents giving rise to structural chromosome aberrations. A clastogen can cause breaks in chromosomes that result in the loss or rearrangement of chromosome segments. Aneugenicity refers to the effects of agents that give rise to a change (gain or loss) in chromosome number. An adequate coverage of all endpoints can only be obtained by the use of multiple test systems (i.e. a testing battery), as no individual test system can cover all endpoints. The standard *in vitro* test battery comprises the Ames test (or bacterial reverse mutation assay), the *in vitro* mammalian cell chromosomal aberration test, the *in vitro* mammalian cell gene mutation test, and the *in vitro* mammalian

cell micronucleus test (see Annex I, Table 1). As a logical choice, any confirmatory *in vivo f*ollow-up test needs to cover the same endpoint as the one which showed positive results *in vitro*.

Generally, the assessment of genotoxic hazard to humans follows a step-wise approach, beginning with a basic battery of *in vitro* tests followed in some cases by *in vivo* testing. Regulatory requirements, in particular for *in vivo* testing, vary depending on the type of chemical under regulation and the region. For cosmetic ingredients and products, *in vivo* testing is prohibited in the EU (EC no 1223/2009) while for industrial chemicals and biocidal products a positive result in one or more of the *in vitro* genotoxicity tests requires confirmation by appropriate follow-up *in vivo* testing (EC no. 1907/2006; EC no. 528/2012). In these cases, if a substance is clearly negative in the *in vitro* battery it is considered as having no genotoxic hazard, thus no further *in vivo* study is needed. Regulatory requirements for pharmaceuticals, veterinary drugs and plant protection products foresee that the *in vitro* testing battery (irrespective of the outcome) is always followed by *in vivo* testing (ICH, 2011; VICH, 2012; EC nos. 1107/2009b and 283/284-2013) (Figure 1). See Annex I for a detailed description of regulatory requirements.

The purpose of this document is to present the EURL ECVAM strategy to improve genotoxicity testing for hazard identification and to either avoid or minimise the use of animals depending on the regulatory context. The EURL ECVAM strategy is based on the state of the science of genotoxicity testing and draws from recent initiatives undertaken by EURL ECVAM and others, including some current guidance documents from the European Food Safety Authority and the UK Committee on Mutagenicity of Chemicals in Food, Consumers Products and the Environment and the International Conference on Harmonization (EFSA Opinion, 2011; UK COM Guidance, 2011; ICH, 2011). An important aspect of this document is to propose solutions that can satisfy information requirements associated with EU legislation and which can also be considered by the Organisation for Economic Co-operation and Development (OECD) in the context of a globally harmonised approach for the assessment of genotoxicity. This strategic plan is intended to be inclusive in that its implementation will rely on the cooperation of EURL ECVAM with other related initiatives and the coordinated contributions from various stakeholders.

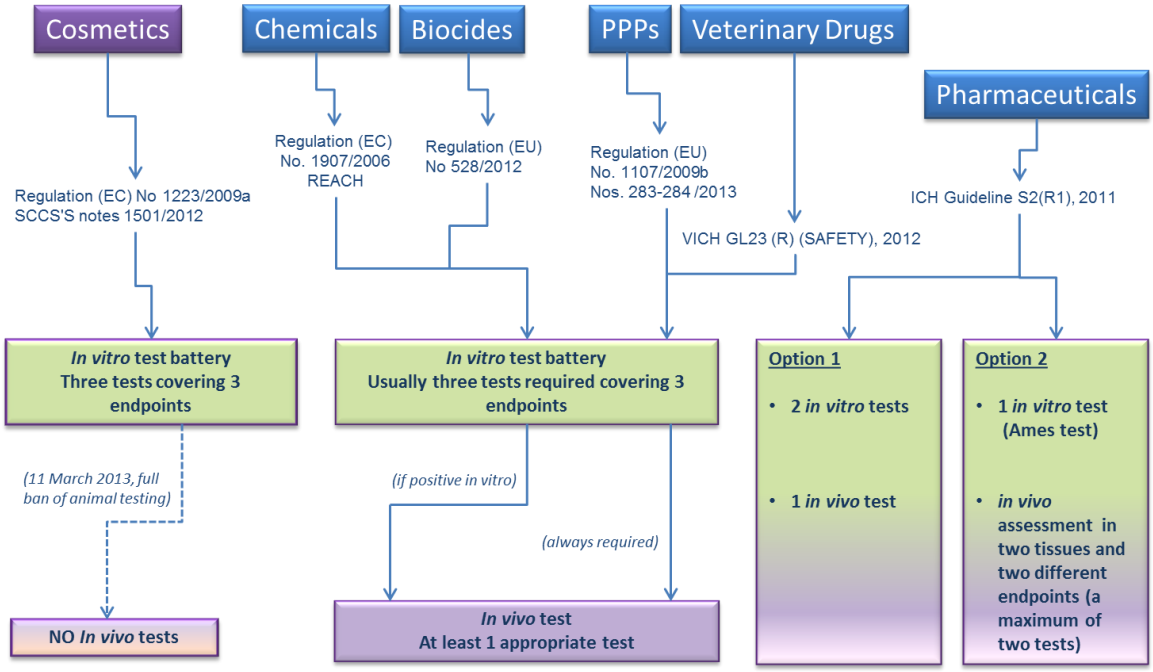

Figure 1. Summary of testing requirements and guidance documents for genotoxicity assessment under different pieces of EU legislation.

2. *In vitro* genotoxicity approaches currently under evaluation

A number of *in vitro* tests are currently used to screen substances for potential genotoxicity, yet, the *in vitro* testing battery cannot at present be considered to fully replace animal tests (Adler et al., 2011). Furthermore, in most sectors confirmatory testing still needs to be carried out *in vivo* in order to comply with regulatory requirements. While these *in vitro* tests have a good sensitivity, some (especially the *in vitro* mammalian cell tests) have a high rate of false positive results (Kirkland et al., 2005; 2007). False positives (also referred as irrelevant or misleading positives) are *in vitro* positive results that cannot be confirmed when followed-up in well-conducted *in vivo* genotoxicity and/or carcinogenicity assays. Thus these positive findings may trigger many unnecessary follow-up *in vivo* confirmatory tests or may even lead to abandoning the further development of promising substances.

Improving existing assays through better understanding

The high rate of misleading positive results in *in vitro* mammalian cell genotoxicity tests was addressed during an ECVAM workshop (Kirkland et al., 2007). It was recommended that better guidance should be developed on the likely mechanisms that lead to positive results but which are not relevant for humans, and on how to obtain evidence that such mechanisms are at play. Some of the reasons identified for false positives outcomes from *in vitro* testing were; excessive cytotoxicity, high cell passage-number, and compromised

p53 response pathways or DNA repair mechanisms in the cells used. For newly developed test methods, cell systems of human origin which are p53 and DNA repair proficient, and which have phase I and phase II metabolism, were considered to offer the best option to reduce false positive results in the future (Kirkland et al., 2007). The recommendations of this workshop have contributed to several international collaborative initiatives (e.g. by Cosmetics Europe, ILSI/HESI, EURL ECVAM, UK NC3Rs and JaCVAM) aiming to improve the existing genotoxicity *in vitro* tests and to identify and evaluate new cell systems with appropriate sensitivity but improved specificity (i.e. reduced false positive rate). For example, EURL ECVAM established a recommended list of genotoxic and non-genotoxic chemicals for assessment of the performance of new and improved genotoxicity tests (Kirkland et al., 2008). This chemical list is used world-wide by test developers and other organisations.

Currently, all OECD Test Guidelines (TG) related to genotoxicity are being revised taking also into account the knowledge acquired during the last decades of testing and the recent activities related to false positives (Parry et al., 2010; Kirkland and Fowler, 2010; Fowler et al., 2012a, 2012b). The recommendations made in the revised TGs both for experimental conditions and data interpretation will most probably enhance the quality of the data that will be produced.

Maintaining the same endpoints but using novel biological models

Several new *in vitro* assays, aiming at confirming positive results in the standard *in vitro* battery or providing additional mechanistic information are under development and evaluation.

The micronucleus test and the comet assay in 3D human reconstructed skin models offer the potential for a more physiologically relevant approach especially regarding metabolic aspects to test dermally applied chemicals (Hu et al., 2010; Pfuhler et al., 2011). It has been anticipated that these features of the reconstructed skin models could improve the predictive value of a genotoxicity assessment compared with that of existing *in vitro* tests and, therefore, could be used as follow-up tests in case of positive results from the standard *in vitro* genotoxicity testing battery (Maurici et al., 2005; Pfuhler et al., 2010). Validation studies of the micronucleus test using the human reconstructed skin models are coordinated and funded by Cosmetics Europe, and in the case of the 3D Skin Comet assay, by a joint effort between Cosmetics Europe and a German Consortium funded by BMBF (Aardema et al., 2010; Reus et al., 2013). These projects also address the integration of an exogenous metabolic activation system that may be required to improve the sensitivity of the assays to detect the genotoxic potential of chemicals activated through liver mediated metabolism, rather than through skin metabolism.

Another promising system that has been proposed as a follow-up for *in vitro* positives is the hen's egg test for micronucleus induction, though it is not obviously a human-based system (HET-MN; Wolf et al., 2008). The HET-MN combines the use of the commonly accepted genetic endpoint “formation of micronuclei” with the well-characterised and complex model of the incubated hen's egg, which enables metabolic activation,

elimination and excretion of xenobiotics, including those that are mutagens or pro-mutagens. The transferability and intra-/inter-laboratory reproducibility are currently being evaluated by a German consortium (Greywe et al., 2012).

Assays based on cell lines and primary cells derived from transgenic rodents hold promise, (Berndt-Weis et al., 2009; Zwart et al., 2012). They can originate from different tissues and are expected to reduce assumptions related to extrapolation from *in vitro* to *in vivo* tests as they assess exactly the same endpoint and marker gene as the respective *in vivo* transgenic models (OECD, 2013).

Methods addressing different endpoints

The *in vitro* cell transformation assay (CTA) has been shown to closely model some key stages of the *in vivo* carcinogenesis process by measuring the transformation of cells (LeBoeuf et al., 1999). The CTA has the potential to detect both genotoxic and non-genotoxic carcinogens. Although it is not a genotoxicity assay, the CTA may be considered as providing additional information to more routinely employed *in vitro* tests and may sometimes be used as a follow-up assay for confirmation of *in vitro* positive results from genotoxicity assays, typically as part of a weight of evidence assessment (Doktorova et al, 2012). Furthermore, data generated by the CTA can be useful where genotoxicity data for a certain substance class have limited predictive capacity (e.g. aromatic amines) (EFSA Opinion, 2011; Vanparys et al., 2012). However, the exact role of the CTA in regulatory toxicology is still under debate. Following the development of an OECD Detailed Review Paper collecting available CTA data and a EURL ECVAM recommendation, an OECD TG on CTA in Syrian hamster embryonic stem cells (SHE) is in development (OECD, Review paper, 2007; EURL-ECVAM, recommendation, 2012; Corvi and Vanparys, 2012; OECD draft TG on SHE CTA method). Another EURL ECVAM recommendation has been drafted on a short-term CTA in the BHAS 42 cell line (Sakai et al., 2011; EURL ECVAM recommendation, 2013).

In the context of genotoxicity testing, the primary use of toxicogenomics-based tests is envisaged to be in providing information on mode of action as supporting evidence (Doktorova et al., 2012). The application of toxicogenomics to predict mode of action has been reviewed in depth (Ellinger-Ziegelbauer et al., 2009; Waters et al., 2010). Although the published *in vitro* and *in vivo* data set show appreciable variability in terms of genes differentially expressed, common features emerge with respect to molecular pathways. For instance, the DNA damage-responsive *p53* pathway is extensively activated both by DNA reactive genotoxins *in vitro* and genotoxic carcinogens *in vivo*. Conversely, *in vitro* DNA non-reactive genotoxins and *in vivo* non-genotoxic carcinogens mostly induce an oxidative stress response, signalling and cell cycle progression genes (Doktorova et al., 2013; Jennings et al., 2013). These data represent a proof of concept that the gene expression profiles reflect the underlying mode of action quite well. However, additional studies should be performed to enlarge the number of chemicals tested and to fill the gaps in dose-response and time-course relationships.

In the last few years several attempts have been made to develop and validate the induction of stress pathways/proteins as endpoints in genotoxicity assays by using high throughput screening approaches. Among these assays are the GreenScreen Human Cells Assay and its further adaptation to use metabolic activation as the Blue Screen Assay, both of which use a p53-competent TK6 lymphoblastoid cell line, genetically modified to incorporate a fusion of the green fluorescent protein or Luciferase reporter-gene and the GADD45 gene, a downstream target of p53 (Hastwell et al., 2006; Birrel et al., 2010; Billinton et al., 2010). Other assays are the high throughput screening luciferase reporter-gene assays based on four different stress pathways (RAD51, Cystatin A p53 and Nrf2) in the HepG2 cell line (Westerink et al., 2010) and the DNA repair-deficient chicken DT40 cell line that measures cytotoxicity by comparing these cells to parental DNA repair-proficient cells (Yamamoto et al., 2011).

Status of current approaches under evaluation and future prospects

Although the *in vitro* methods mentioned above are indeed promising, they are not mature enough to replace the current regulatory assays. However they can already be used to better understand mechanisms, judge the relevance of the data obtained with the standard assays (e.g. differentiating DNA-reactive from DNA-non-reactive compounds), and to help predict *in vivo* effects when animals cannot be used.

Other emerging technologies (Lynch et al., 2011) and non-classical methodologies for the evaluation of genotoxicity may ultimately drive the development of completely new integrated approaches to testing and assessment, which break away from the paradigm established over the past 40 years of regulatory testing. However considerable research and development effort supported by ample investment is still required before more 'radical' solutions emerge which can stand up to the rigorous demands of regulatory testing. In the short to medium term therefore, reduction and possibly replacement of animal testing for genotoxicity assessment is most likely achievable through a pragmatic approach of using sound scientific rationale to improve the current testing paradigm, in a manner acceptable to both the regulatory and regulated communities.

3. Strategic plan to avoid and reduce animal use in genotoxicity testing

EURL ECVAM proposes that efforts should be directed towards the overall improvement of the current genotoxicity testing strategy for better hazard assessment with the use of no or fewer animals. Moreover, the intention is to demonstrate that the improved testing strategy is adequate to satisfy the information requirements of various EU regulations. The EURL ECVAM strategy involves the pursuit of two key aims, as follows;

Strategic Aim 1 Enhance the performance of the *in vitro* testing battery to reduce the need for *in vivo* follow-up tests.

Strategic Aim 2 Reduce and optimise the use of animals during *in vivo* testing.

Several opportunities for achieving these aims and improving the testing strategy have been identified (**Figure 2**), the realisation of which will have significant impact on regulatory testing in different industrial sectors. This includes helping the cosmetics industry to assess ingredients without *in vivo* testing, reducing the number of *in vivo* genotoxicity tests required under other legislation, and eventually reducing the need for *in vivo* carcinogenicity testing (**Figure 3**). Pursuing this strategy will initially require exploiting the current standard test methods and all available data, while in certain cases this may trigger further experimental investigations.

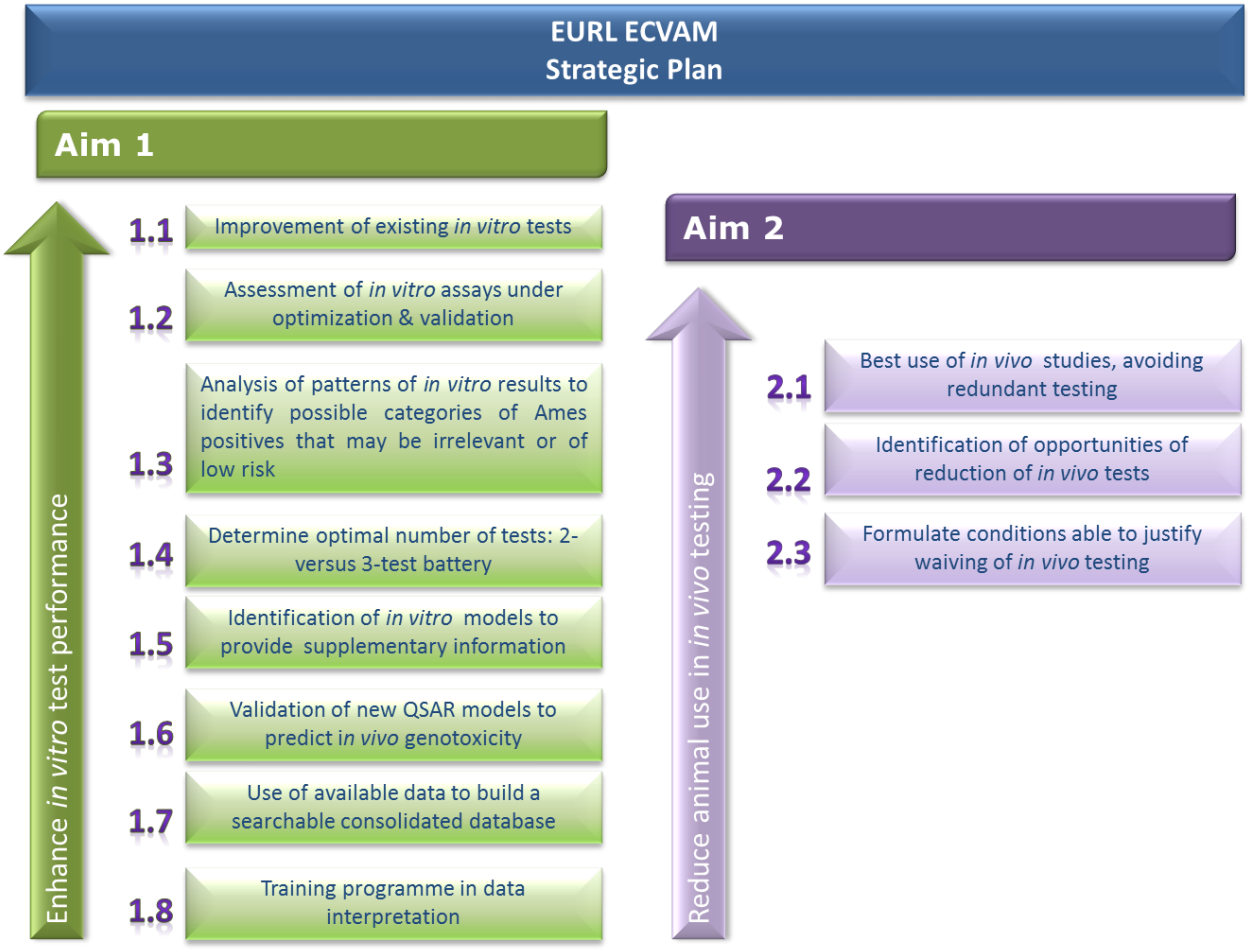

Figure 2. EURL ECVAM strategic plan to avoid or minimise animal use in genotoxicity testing.

3.1. Strategic Aim 1: Enhance the performance of the *in vitro* testing battery to reduce the need for *in vivo* follow-up tests

Analysis of the most suitable combinations of *in vitro* genotoxicity tests is a key consideration for possible improvements of the testing strategies for genotoxicity. This applies both to the base set *in vitro* testing battery and to supplementary *in vitro* tests used to confirm the relevance of positive results *in vitro*.

Objective 1.1. Improvement of existing in vitro tests

In recent years many activities have been carried out in trying to understand how to reduce false positive results in the *in vitro* mammalian cell tests (Fellows et al., 2008; Parry et al., 2010; Kirkland and Fowler, 2010; Fowler et al., 2012a, 2012b). It is now the opportune time to assess what has been achieved so far and what still needs to be done.

Short-term goal

- Review the state of art to identify if there are still areas for improvement of quality and relevance of the assays and if new questions have arisen from recent initiatives.

Objective 1.2. Determine the optimal number of tests for the standard in vitro battery

Recently, the EFSA Scientific Committee and the UK Committee on Mutagenicity of Chemicals in Food, Consumers Products, and the Environment (COM) recommended that the set of tests used in the current *in vitro* battery be as small as scientifically justifiable in order to reduce the number of false positives (EFSA Opinion, 2011; COM Guidance, 2011). Based on a recent data analysis (Kirkland et al., 2011), a two-test initial step has been proposed which includes the Ames test (OECD TG 471) and the *in vitro* micronucleus test (OECD TG 487). As confirmed by a previous ECVAM retrospective validation study, the *in vitro* micronucleus test detects two of the endpoints (i.e. structural and numerical chromosome aberration; Corvi et al., 2006), thus the proposed two-test battery covers all three endpoints to be assessed in the standard testing battery.

Medium-term goal

- Promote the implementation of the two-test battery approach within the international regulatory community for different sectors through dialogue and round-table discussions among regulatory organisations, agencies and committees.

Objective 1.3. Assessment of in vitro genotoxicity assays currently under optimisation and evaluation

Several new *in vitro* test systems exist (described in section 3), which are at different stages of optimisation and evaluation. In particular, some of these tests are good candidates to follow up positive results from the standard battery of tests (e.g. micronucleus and comet assay in reconstructed skin models).

Medium-term goals

- Finalisation of validation and peer review of the test methods currently under evaluation.
- Contingent upon the outcome of validation, identify best ways to exploit those methods within the testing strategy.

Objective 1.4. Identification of in vitro tests that provide supplementary information

Further *in vitro* tests may be considered as part of follow-up testing for *in vitro* positive genotoxic results to generate supplementary information regarding mechanism (or mode) of action and to clarify the relevance of the positive results for humans. For example, *in vitro* tests that measure gene expression may be considered particularly useful.

In addition, since different tissues may be the target of different types of compounds, it should be explored whether *in vitro* tests could provide reliable and sufficient information on local genotoxicity, rather than developing further *in vivo* tests. Based on the metabolic characterisation of these test systems and available data on kinetics, their usefulness in extrapolation to systemic and germ cell genotoxicity should be determined.

Short-term goal

- Identify useful mechanistic *in vitro* tests to help with the decision-making process.

Medium-term goals

- Mapping of circumstances in which such tests are needed and which ones should be applied.
- Identify and assess *in vitro* tests that may be predictive of site-of-contact genotoxicity and characterise their metabolic capacity.

Objective 1.5. Analysis of different patterns of in vitro results to identify possible categories of positive results from the Ames test that may be irrelevant or signify a low risk

The Ames test conducted in bacteria is the most commonly used genotoxicity test within the *in vitro* battery as it is considered able to reveal DNA reactivity and DNA reactive compounds. It is used to assess almost all types of substances including impurities, low production volume chemicals, etc. Therefore, knowing whether *in vitro* positive results are accurate indicators of *in vivo* mutagenic potential or carcinogenicity is extremely important in determining whether follow-up *in vivo* tests are needed or whether substances should be further tested. Despite the many activities on false positive results in *in vitro* mammalian cell tests, positive results in the Ames test have not been analysed in the same way as for mammalian cell tests. In this context, EURL ECVAM recently held a workshop and initiated a project with the aim of 1) evaluating the predictivity of the Ames for *in vivo* genotoxicity and carcinogenicity when considered alone or in association with a mammalian cell assay for the detection of chromosome damage and/or gene mutations, and 2) better characterising the cases where the Ames test seems to lead to irrelevant (false positive) results (e.g. chemical classes, type of bacterial strains, magnitude of effects). As data presented at the workshop were from different sources (ECHA dissemination

database, EFSA pesticides, carcinogens of the Carcinogenicity and Genotoxicity eXperience database (CGX), US NTP, US FDA, NTP, FDA, Japanese CSCL and ISHL, SCCS and other industry databases), it was recommended that a consolidated database be constructed.

Short-term goal

- Construct a consolidated database of Ames positive compounds with respective available *in vitro* and *in vivo* genotoxicity and carcinogenicity data.

Medium-term goal

- Determine if all types of Ames positive test results predict a similar level of genotoxic and carcinogenic hazard.

Objective 1.6. Construct a consolidated genotoxicity database

Gathering reliable information from genotoxicity testing which is spread across several databases is demanding and often time consuming.

The generation of a reliable consolidated database, which would include information derived from different existing databases in a harmonised format will be useful for future analyses and further mechanistic understanding. Inconsistency (contradictory data derived from different sources) and poor quality of the data (i.e. data not compliant with the current state of the art) will be the main issues to be addressed in such an exercise. The starting point for this activity will be the consolidated database of Ames positive results.

Short-term goal

- Promote a data sharing exercise for non-publicly available data.

Medium-term goal

- Construct a consolidated public database of genotoxicity data.

Objective 1.7. Training programme in data interpretation

Uncertainties in the interpretation of data from *in vitro* tests and appropriate use of weight of evidence arguments among inexperienced assessors may often lead to the request of unnecessary follow-up animal studies.

Short-term goal

- Initiate a training programme addressing *in vitro* data interpretation.

Objective 1.8. QSAR as a tool to support weight-of-evidence assessments of genotoxicity

QSAR models can be used alongside the *in vitro* battery to provide additional information in weight-of-evidence assessments of genotoxicity. Many QSAR models have been published in the literature for predicting *in vitro* genotoxicity and the most commonly modelled test has been the Ames test. Numerous studies have characterised the predictive performances of these models (e.g. Worth et al., 2010; Hilbrecht et al., 2011; Bakhytari et

al., 2013), including work carried out in the context of projects funded by the European Commission (EC), e.g. OSIRIS (http://www.ufz.de/Osiris) and ANTARES (http://www.antares-life.eu/), and by EFSA (http://www.efsa.europa.eu/en/supporting/pub/50e.htm). The applicability of some of these models, and their integration with other non-testing approaches such as read-across, is being explored within the EC-funded CALEIDOS project (http://www.caleidos-life.eu).

In contrast to *in vitro* genotoxicity, relatively few QSAR models have been developed to predict *in vivo* genotoxicity. A decision tree based on structural alerts for the *in vivo* micronucleus test (Benigni et al, 2010) has been implemented in the freely available software tools, Toxtree and the OECD Toolbox. In addition, a commercially available tool, TIMES, explicitly takes metabolic pathways into account (Mekenyan et al., 2012). To support further QSAR development, a database of *in vivo* micronucleus data has been developed and made publicly available (Benigni et al., 2012).

Short-term goal

- Evaluate the status of QSAR methods for *in vivo* genotoxicity prediction, and identify opportunities for further model development.

Medium-term goal

- Explore the integrated use of QSAR models and *in vitro* tests in genotoxicity assessment.

3.2. Strategic Aim 2: Reduce and optimise the use of animals during *in vivo* testing

Since *in vivo* genotoxicity tests are still required to confirm positive *in vitro* results in most regulatory contexts, it is important to reduce and optimise the use of animals during *in vivo* follow-up tests and so avoid performing unnecessary animal tests as far as possible.

Objective 2.1. Achieve best use of in vivo tests avoiding redundant testing

The selection of the appropriate follow-up test is particularly important in view of the adoption of new *in vivo* genotoxicity OECD TGs (OECD TG 488 on transgenic mice and draft OECD TG on *in vivo* comet assay). New *in vivo* tests have been developed in order 1) to be able to evaluate genotoxicity in almost all tissues, and 2) to measure end-points other than chromosome damage. However, with an increased number of *in vivo* tests available, there is a risk that more animals could be used in the future for the assessment of genotoxicity. The choice of the *in vivo* follow-up test needs to cover the same endpoint as the one which showed positive results *in vitro*. With the objective of reducing the use of experimental animals according to the 3Rs principles, as a default only one *in vivo* test should be performed initially. It is recommended that this test covers different endpoints in order to attain maximum data using the minimum number of animals whilst ensuring that animal welfare is not compromised further. A second *in vivo* test should only be

considered if the first *in vivo* test is negative, but does not properly cover all *in vitro* positive genotoxic endpoints (Dearfield, 2011). For substances that are genotoxic *in vivo* the potential to affect germ cells should be investigated mainly based on toxicokinetics considerations. If it is reasonable to conclude that the substance can cause heritable genetic damage from available information, then the substance can be considered a somatic and germ cell mutagen and no further testing should be performed.

Short-term goal

- Develop general guidance on above issues. Work is already on-going under the umbrella of the OECD considerations on Animal Welfare (Mutual Acceptance of Data Act, 1981; OECD, Guidance Documents, nos. 19/2000 and 34/2005).

Medium-term goal

- Develop clear guidance on the most appropriate choice of *in vivo* test to be used within a testing strategy under different scenarios, ultimately leading to better assessments with fewer animals.

Objective 2.2. Identification of opportunities for reduction of in vivo tests

Several opportunities for reduction were discussed during an ECVAM workshop (Pfuhler et al., 2009). These opportunities exist both at single test level (e.g. 1 sex versus 2 sexes, omission of positive control) and ITS level (e.g. integration of endpoints in the same animals) and need to be taken into consideration whenever possible (EFSA Opinion, 2011). Some of these opportunities are already available and compliant from the regulatory point of view, but are not always implemented. Combination of different endpoints into a single study or incorporation of scientifically appropriate *in vivo* genotoxicity endpoints into a short-term repeated dose toxicity test (28 days) (Pfuhler et al., 2009; Bowen et al., 2011; Rothfuss et al., 2010, 2011; EFSA Opinion, 2011), if such a test is going to be performed anyhow, should always be considered. Most of the present genotoxicity *in vivo* tests (e.g. micronucleus test, comet assay, and the new Pig-a gene mutation assay) are amenable to such combination and integration. An integration of genotoxicity endpoints in the repeated dose toxicity study, when the top dose is appropriate (ICH S2(R1), 2011) offers the possibility for an improved interpretation of genotoxicity findings since such data will be evaluated in conjunction with other routine toxicological information obtained, such as haematology, clinical chemistry and histopathology also in the context of exposure (Pfuhler et al., 2009). Maximising the information that can be extracted from a single test (e.g. endpoint combination, analysis of different tissues, proof of exposure) can reduce the overall number of assays required and thus ultimately lead to a reduction of animals used.

Short-term goals

- Review of progress since last ECVAM workshop on reduction opportunities to identify further needs, especially in relation to integration of genotoxicity endpoints in the *in vivo* 28-day repeated dose toxicity test; combination of different genotoxicity endpoints (e.g. micronucleus and comet) in the same animal test; omission of positive controls.

- Investigate proof of exposure in the treated animals by e.g. microsampling techniques, such as use of dried blood spots sampling. This would avoid carrying out additional toxicokinetic studies. This is already under investigation by several organizations (e.g. NC3Rs).

Objective 2.3. Formulate conditions which justify waiving of in vivo testing

In certain cases, there may be conditions where a conscious decision not to do *in vivo* genotoxicity tests can be made. For chemicals and biocides, this may be the case on absence of or low exposure, knowledge about structurally similar chemicals (read-across and grouping) or where it can be sufficiently deduced that an *in vitro* positive finding is not relevant for *in vivo* situations. For pharmaceuticals, plant protection products and veterinary drugs where an *in vivo* test is always part of the standard battery, knowledge of the known biological effects and the modes of action of the compounds may lead to an estimate of the probability that the substance is a genotoxic carcinogen.

Medium-term goals

- Define conditions where positive *in vitro* tests are likely to not provide evidence for a possible genotoxicity hazard to humans.
- Provide recommendations for the regulatory community on the criteria to omit *in vivo* genotoxicity testing.
- Demonstrate how reliance on clear positive genotoxicity results can be used as a basis for classification of carcinogens in category 1B or 2, without the need to perform a carcinogenicity bioassay (e.g. for REACH). This needs careful consideration on what additional information would be necessary to come to such conclusions.

4. Evaluation of test methods submitted to EURL ECVAM

EURL ECVAM will continue evaluating submitted *in vitro* test methods in the light of this strategic plan. Such evaluations typically conclude on the status of a method with regard to its reliability and relevance, based on the information available, and advise on whether it should be considered for validation and/or peer-review and perhaps submission to the OECD as the basis for Test Guideline development (see for further information: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam). In light of this EURL ECVAM strategy therefore, the assessment of incoming methods will be based both on their own merits and on the value of information they provide with respect to the methods currently in use, and to the gaps that have been identified in the current testing strategy. In addition, new methods that provide equivalent information to existing methods will also be considered in terms of possible advantages related to ease of use, cost, throughput and widespread availability.

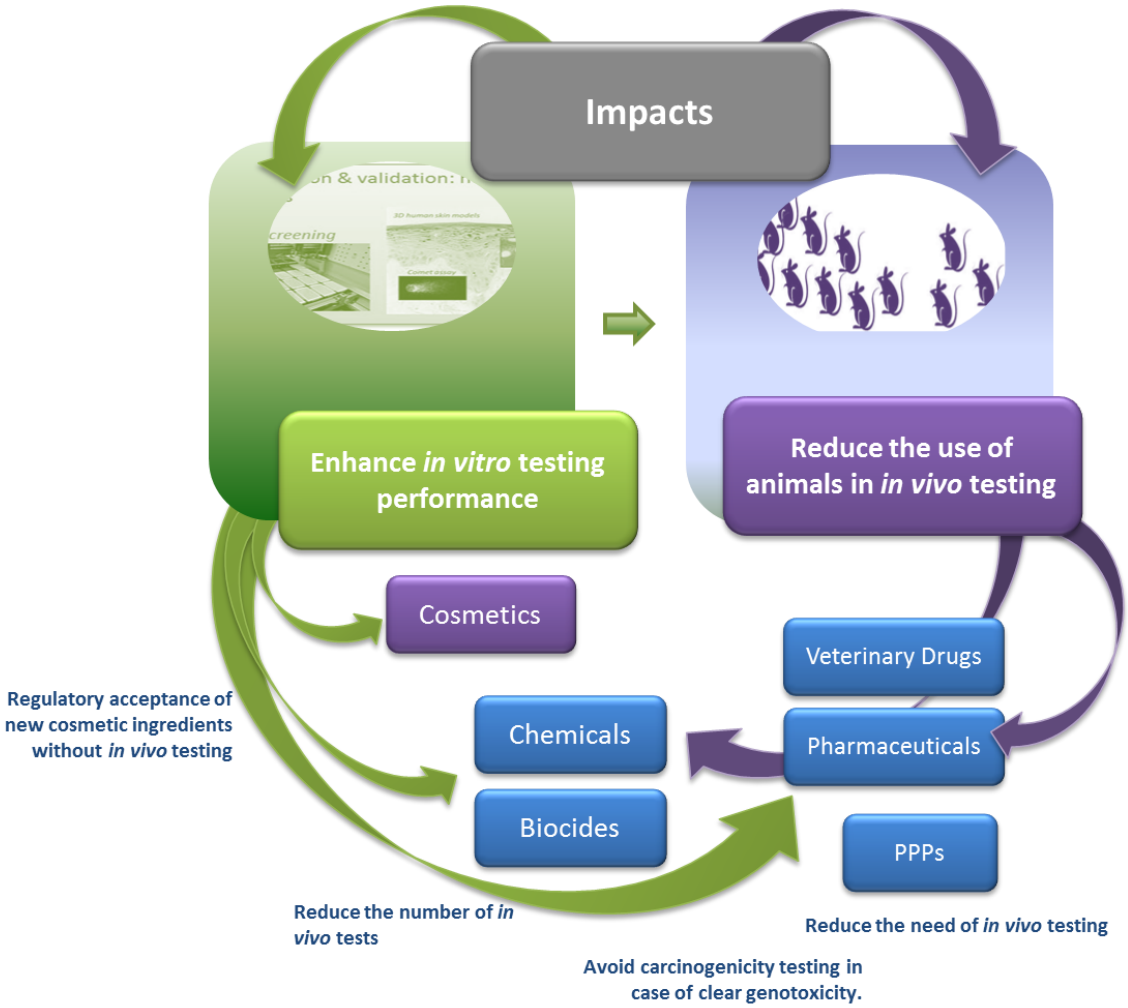

Figure 3. Possible impact on regulatory testing in different sectors upon realization of the strategy

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Annex

Regulatory Requirements for Genotoxicity/Mutagenicity

The following sections illustrate the information requirements and guidance documents for genotoxicity/mutagenicity within the legislation considered for the purpose of this report (**Figure 1**). The assessment of genotoxic hazard to humans follows in general a step-wise approach; starting with a battery of *in vitro* tests followed in some cases by *in vivo* tests (see **Table 1** for regulatory tests). Regulatory requirements vary depending on the type of substance under regulation and the region, especially for *in vivo* testing. Quotations from the original legal text and/or guidelines are reported in *italics*).

Table 1. Available *in vitro* and *in vivo* methods for genotoxicity/mutagenicity.

Test Method	COUNCIL REGULATION (EC) No 440/2008	OECD Test Guideline	Endpoint	*in vitro/ in vivo*
Bacterial reverse mutation test (Ames test)	B.13-14	TG 471[a]	Gene mutations	*In vitro*
In vitro Mammalian chromosome aberration test	B.10	TG 473[a]	Structural aberrations	*In vitro*
In vitro Mammalian cell gene mutation test	B.17	TG 476[a,b]	Gene mutations and structural chromosome aberrations	*In vitro*
In vitro Mammalian cell micronucleus test	B.49	TG 487[a]	Structural and numerical aberrations	*In vitro*
Mammalian erythrocyte micronucleus test	B.12	TG 474[a]	Structural and numerical aberrations	*In vivo*
Mammalian bone marrow chromosome aberration test	B.11	TG 475[a]	Structural aberrations	*In vivo*
Transgenic rodent somatic and germ cell gene mutation assays	--	TG 488	Gene mutations	*In vivo*
Unscheduled DNA Synthesis (UDS) test with mammalian liver cells	B.39	TG 486	DNA repair activity	*In vivo*
Rodent Comet assay	--	*Draft TG*	DNA damage	*In vivo*
Rodent Dominant Lethal test	B.22	TG 478	Germ cell mutagenicity: structural and numerical chromosome aberrations	*In vivo*
Mammalian Spermatogonial Chromosome Aberration test	B.23	TG 483	Germ cell mutagenicity: structural chromosome aberrations	*In vivo*
Mouse Heritable Translocation assay	B.25	TG 485	Germ cell mutagenicity: structural and numerical chromosome aberrations	*In vivo*
Syrian Hamster Embryo (SHE) cell transformation assay	--	*Draft TG*[d]	Cell transformation	*In vitro*

[a]Most commonly used methods in regulatory genotoxicity assessment; [b]TG 476 is under revision and two distinct guidelines are in preparation: one for *hprt* locus mutations detection and a second for *tk* locus. [c]Structural chromosome aberration can be detected in the *tk* test. [d]This is not a genotoxicity test, but it is considered a test to assess carcinogenic potential in vitro.

OECD TGs are available at:
http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

1. Chemicals

The REACH Regulation (EC) No. 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals and the CLP Regulation (EC) No. 1272/2008 are closely interlinked to each other, since the classification of a Chemical is a mandatory part of the REACH registration process.

1.1. REACH Regulation

The REACH Regulation (EC) No. 1907/2006 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 is dependent on the tonnage band of a substance.

The first REACH deadline for registering substances manufactured or imported at 1000 tonnes or more per year, carcinogenic, mutagenic or toxic to reproduction substances (CMRs) above 1 tonne per year and suspected persistent, bioaccumulative and toxic substances (*PBTs*), very persistent and very bioaccumulative substances (*vPvBs*) above 100 tonnes per year was on 30 November 2010. By that deadline 4300 registration dossiers were successfully submitted to ECHA, including nearly 3400 phase-in substances.

The REACH deadline for registering substances manufactured or imported in quantities of 100 to 1000 tonnes per year was on 31 May 2013. By that time, 3215 companies had submitted 9084 registration dossiers to ECHA, consisting of 2998 unique phase-in substances (http://echa.europa.eu/web/guest/reach-2013; Accessed on September 10, 2013).

Phase-in substances manufactured or imported in volumes of 1-100 tonnes per year will need to be registered by the 31st May 2018.

According to a report prepared by the former European Chemicals Bureau of the JRC it was estimated that a high percentage of the phase-in substances (**Figure 4**) would have to undergo further in vivo genotoxicity/mutagenicity testing (Van der Jagt et al., 2004).

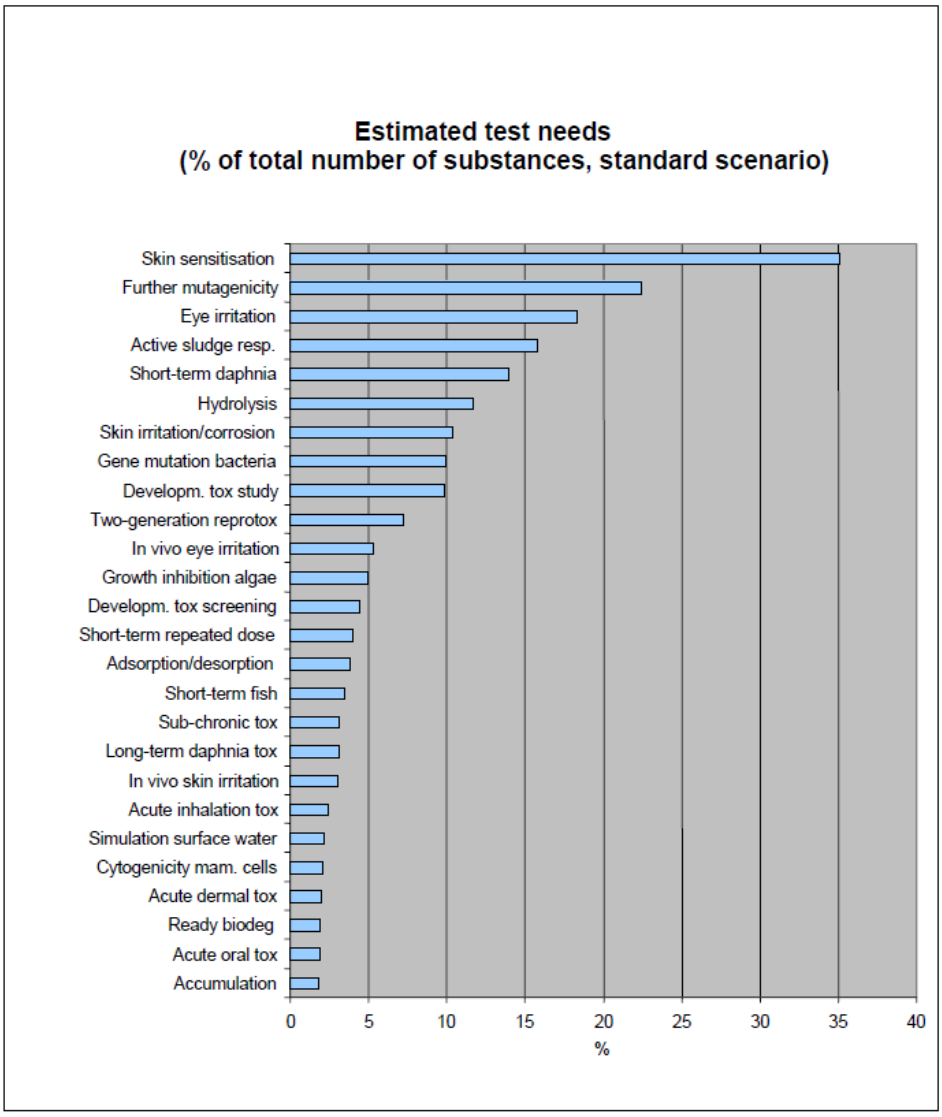

Figure 4. Estimated percentage of the total number of phase-in substances in need of being tested for the different toxicological endpoints under REACH (Van der Jagt et al. 2004, EUR 21405 EN).

Standard information requirements for substances manufactured or imported in quantities of:

- *Annex VII (1 tonne or more)*
  Mutagenicity [par. 8.4]
  In vitro gene mutation study in bacteria.
  Further mutagenicity studies shall be considered in case of a positive result.

- *Annex VIII (10 tonnes or more)*
  i. ***Mutagenicity*** *[par. 8.4]*

ii. ***In vitro cytogenicity study in mammalian cells or in vitro micronucleus study****. [par. 8.4.2] The study does not usually need to be conducted – if adequate data from an in vivo cytogenicity test are available or – the substance is known to be carcinogenic category 1 or 2 or mutagenic category 1, 2 or 3 (referring to GHS categories 1A, 1B and 2, respectively).*

iii. ***In vitro gene mutation study in mammalian cells*** *[par. 8.4.3], if a negative result in Annex VII and Annex VIII. The study does not usually need to be conducted if adequate data from a reliable in vivo mammalian gene mutation test are available. Appropriate in vivo mutagenicity studies shall be considered in case of a positive result in any of the genotoxicity studies in Annex VII or VIII.*

- *Annex IX (100 tonnes or more)*

  Specific rules for adaptation from Column 1 *[par. 8.4]*

  If there is a positive result in any of the in vitro genotoxicity studies described in Annex VII or VIII and there are no results available from an in vivo study already, an appropriate in vivo somatic cell genotoxicity study shall be proposed by the registrant.

  If there is a positive result from an in vivo somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered.

- *Annex X (1000 tonnes or more)*
  Specific rules for adaptation from Column 1 *[par. 8.4]*

  If there is a positive result in any of the in vitro genotoxicity studies described in Annexes VII or VIII, a second in vivo somatic cell test may be necessary, depending on the quality and relevance of all the available data.
  If there is a positive result from an in vivo somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered.

1.2. CLP Regulation

The CLP Regulation 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 Harmonized System (GHS) (UN 2011), ST/SG/AC.10/30/Rev.4 United Nations [Chapter 3.5].

Definitions and general conditions [par. 3.5.1.]

A mutation means a permanent change in the amount or structure of the genetic material in a cell. The term 'mutation' applies both to heritable genetic changes that may be manifested at the phenotypic level and to the underlying DNA modifications when known (including specific base pair changes and chromosomal translocations). The term 'mutagenic' and 'mutagen' will be used for agents giving rise to an increased occurrence of mutations in populations of cells and/or organisms.

The more general terms 'genotoxic' and 'genotoxicity' apply to agents or processes which alter the structure, information content, or segregation of DNA, including those which cause DNA damage by interfering with normal replication processes, or which in a non-physiological manner (temporarily) alter its replication. Genotoxicity test results are usually taken as indicators for mutagenic effects.

Classification criteria for substances [par. 3.5.2.]

This hazard class is primarily concerned with substances that may cause mutations in the germ cells of humans that can be transmitted to the progeny. ***However, the results from mutagenicity or genotoxicity tests in vitro and in mammalian somatic and germ cells in vivo are also considered in classifying substances and mixtures within this hazard class****.*

Hazard categories for germ cell mutagens [Table 3.5.1]

Category 1: Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans.

Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans.

Category 1B is based on:

- *positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or*
- *positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells.*

It is possible to derive this supporting evidence from:

- *mutagenicity/genotoxicity tests in germ cells in vivo, or*
- *by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or*
- *positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.*

Category 2: Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans.

- positive evidence obtained from experiments in mammals ***and/or in some cases from in vitro experiments, obtained from****:*

a) somatic cell mutagenicity tests in vivo, in mammals; or

b) other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.

2. Cosmetics

The Regulation (EC) No. 1223/2009 establishes rules to be complied with by any cosmetic product made available on the market, in order to ensure the functioning of the internal market and a high level of protection of human health. From **11 July 2013**, this Regulation has replaced the Cosmetics Directive 76/768/EEC and following Amendments (Directive 93/35/EEC, Directive 2003/15/EC).

The Regulation also prohibits the placing on the European Union market of *[Chapter V, Article 18, Animal testing]*:

- Products where the final formulation has been the subject of animal testing;
- Products containing ingredients or combinations of ingredients which have been the subject of animal testing.

Based on the recent SCCS's Notes of Guidance (SCCS/1501/12), which is currently under revision, three assays, for the basic level testing of cosmetic substances are recommended:

1. Tests for gene mutation:

i) Bacterial Reverse Mutation Test [EC B.13/14, OECD 471]
ii) In vitro Mammalian Cell Gene Mutation Test [EC B.17, OECD 476]

2. Tests for clastogenicity and aneugenicity

i) In vitro Micronucleus Test [EC B.49, OECD 487]
or
ii) In vitro Mammalian Chromosome Aberration Test [EC B.10, OECD 473]

- *In cases where negative results are seen in the conducted tests, a mutagenic potential is excluded.*
- *Likewise, in cases where a positive result is seen in one of the tests, the compound has to be considered as a (in vitro/intrinsic) mutagen.*

At present no validated methods are available that allow the follow-up of positive results from standard *in vitro* assays [SCCP/1212/09].

3. Biocides

Data requirement of the Regulation (EU) No. 528/2012 are reported as follows:

- ***Mutagenicity*** *[par. 8.5] The assessment of this endpoint shall comprise the following consecutive steps:*

    i. *An assessment of the available in vivo genotoxicity data.*
    ii. *An in vitro test for gene mutations in bacteria, an in vitro cytogenicity test in mammalian cells and an in vitro gene mutation test in mammalian cells are required.*
    iii. *Appropriate in vivo genotoxicity studies shall be considered in case of a positive result in any of the in vitro genotoxicity studies.*

- ***In vivo genotoxicity study*** *[par. 8.6] The assessment of this endpoint shall comprise the following consecutive steps:*
    i. *If there is a positive result in any of the in vitro genotoxicity studies and there are no results available from an in vivo study already, an appropriate in vivo somatic cell genotoxicity study shall be proposed/conducted by the applicant.*
    ii. *If either of the in vitro gene mutation tests is positive, an in vivo test to investigate unscheduled DNA synthesis shall be conducted.*
    iii. *A second in vivo somatic cell test may be necessary, depending on the results, quality and relevance of all the available data.*
    iv. *If there is a positive result from an in vivo somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence to demonstrate that the substance reached the tested organ. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered.*

    Additional Data Set (ADS) The study/ies do (es) not generally need to be conducted if:

    i. *The results are negative for the three in vitro tests and if no metabolites of concern are formed in mammals or,*
    ii. *Valid in vivo micronucleus data is generated within a repeat dose study and the in vivo micronucleus test is the appropriate test to be conducted to address this information requirement.*
    iii. *The substance is known to be carcinogenic category 1A or 1B or mutagenic category 1A, 1B or 2.*

4. Plant Protection Products

The Regulation (EC) No. 1107/2009 establishes rules to be complied with by any plant protection product made available on the market. An active substance, safener or synergist shall only be approved if, on the basis of assessment of higher tier genotoxicity testing carried out in accordance with the data requirements for the active substances, safeners or synergists and other available data and information, including a review of the scientific literature, reviewed by the Authority, it is not or it has not to be classified, in accordance with the provisions of CLP Regulation (EC) No 1272/2008, as mutagen category 1A or 1B.

Genotoxicity testing *[par. 5.4]*
Requirements of the 2013 Update of the Regulation, (EU) No 283/2013 and (EU) No 284/2013:
The aim of genotoxicity testing shall be to:

- *predict genotoxic potential,*
- *identify genotoxic carcinogens at an early stage,*
- *elucidate the mechanism of action of some carcinogens.*

Appropriate dose levels, depending on the test requirements, shall be used in either in vitro or in vivo assays. A tiered approach shall be adopted, with selection of higher tier tests being dependent upon interpretation of results at each stage.

Special testing requirements in relation to photomutagenicity may be indicated by the structure of a molecule. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance and its major metabolites is less than 1 000 L × mol –1 × cm –1, photomutagenicity testing is not required.

In vitro studies *[par. 5.4.1]*
The following in vitro mutagenicity tests shall be performed:

i. *bacterial assay for gene mutation (Ames test),*
ii. *combined test for structural and numerical chromosome aberrations in mammalian cells,*
iii. *test for gene mutation in mammalian cells*

Additional
However, if gene mutation and clastogenicity/aneuploidy are detected in a battery of tests consisting of Ames and in vitro micronucleus (IVM), no further in vitro testing needs to be conducted.

If there are indications of micronucleus formation in an in vitro micronucleus assay further testing with appropriate staining procedures shall be conducted to clarify if there is an aneugenic or clastogenic response. Further investigation of the aneugenic response may be considered to determine whether there is sufficient evidence for a threshold mechanism and

threshold concentration for the aneugenic response (particularly for non-disjunction).

Active substances which display highly bacteriostatic properties as demonstrated in a range finding test shall be tested in two different in vitro mammalian cell tests for gene mutation. Non performance of the Ames test shall be justified.

For active substances bearing structural alerts that have given negative results in the standard test battery, additional testing may be required if the standard tests have not been optimised for these alerts. The choice of additional study or study plan modifications depends on the chemical nature, the known reactivity and the metabolism data on the structurally alerting active substance.

In vivo studies in somatic cells *[5.4.2]*

If all the results of the in vitro studies are negative, at least one in vivo study shall be done with demonstration of exposure to the test tissue (such as cell toxicity or toxicokinetic data), unless valid in vivo micronucleus data are generated within a repeat dose study and the in vivo micronucleus test is the appropriate test to be conducted to address this information requirement.
A negative result in the first in vivo test in somatic cells shall provide sufficient reassurance for active substances that are negative in the three in vitro tests.

Additional
For active substances for which an equivocal or a positive test result is obtained in any in vitro test, the nature of additional testing needed shall be considered on a case-by-case basis taking into account all relevant information using the same endpoint as in the in vitro test.

If the in vitro mammalian chromosome aberration test or the in vitro micronucleus test is positive for clastogenicity, an in vivo test for clastogenicity using somatic cells such as metaphase analysis in rodent bone marrow or micronucleus test in rodents shall be conducted.

If the in vitro micronucleus test for numerical chromosome aberrations on mammalian cells is positive or the in vitro mammalian chromosome test is positive for numerical chromosome changes, an in vivo micronucleus test shall be conducted. In case of positive result in the in vivo micronucleus assay, appropriate staining procedure such as fluorescence in-situ hybridisation (FISH) shall be used to identify an aneugenic and/or clastogenic response.

If either of the in vitro gene mutation tests is positive, an in vivo test to investigate the induction of gene mutation shall be conducted, such as the Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay.

When conducting in vivo genotoxicity studies, only relevant exposure routes and methods (such as admixture to diet, drinking water, skin application, inhalation and gavage) shall be used. There shall be convincing evidence that the relevant tissue will be reached by the chosen exposure route and application method. Other exposure techniques (such as intraperitoneal or subcutaneous injection) that are likely to result in abnormal kinetics, distribution and metabolism shall be justified.
Consideration shall be given to conducting an in vivo test as part of one of the short-term toxicity studies (described under point 5.3)

In vivo studies in germ cells *[par. 5.4.3]*

The necessity for conducting these tests shall be considered on a case by case basis, taking into account information regarding toxicokinetics, use and anticipated exposure.

For most of the active substances recognised as in vivo somatic cell mutagens no further genotoxicity testing shall be necessary since they will be considered to be potential genotoxic carcinogens and potential germ cell mutagens.

Additional
However, in some specific cases germ cells studies may be undertaken to demonstrate whether a somatic cell mutagen is or is not a germ cell mutagen.

The type of mutation should be also considered when selecting the appropriate test. A study for the presence of DNA adducts in gonad cells may also be considered.

5. Pharmaceuticals

The standard test battery for genotoxicity consists of two equally suitable Options as reported in the S2 (R1) ICH Guideline *[Chapter 2]*:

- *Option 1*
  i. *A test for gene mutation in bacteria.*
  ii. *A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma Tk gene mutation assay.*
  iii. *An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, or for micronuclei or for chromosomal aberrations in metaphase cells.*

- *Option 2*
  i. *A test for gene mutation in bacteria.*
  ii. *An in vivo assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay. Typically this would be a DNA strand breakage assay in liver, unless otherwise justified.*

Under both standard battery options, either acute or repeat dose study designs in vivo can be used. In case of repeated administrations, attempts should be made to incorporate the genotoxicity endpoints into toxicity studies, if scientifically justified. When more than one endpoint is evaluated in vivo it is preferable that they are incorporated into a single study. Often sufficient information on the likely suitability of the doses for the repeat-dose toxicology study is available before the study begins and can be used to determine whether an acute or an integrated test will be suitable.

For compounds that give negative results, the completion of either option of the standard test battery, performed and evaluated in accordance with current recommendations, will usually provide sufficient assurance of the absence of genotoxic activity and no additional tests are warranted. Compounds that give positive results in the standard test battery might, depending on their therapeutic use, need to be tested more extensively.

There are several in vivo assays that can be used as the second part of the in vivo assessment under option 2, some of which can be integrated into repeat-dose toxicology studies. The liver is typically the preferred tissue because of exposure and metabolizing capacity, but choice of in vivo tissue and assay should be based on factors such as any knowledge of the potential mechanism, of the metabolism in vivo, or of the exposed tissues thought to be relevant.

Information on numerical changes can be derived from the mammalian cell assays in vitro and from the micronucleus assays in vitro or in vivo. Elements of the standard protocols that can indicate such potential are elevations in the mitotic index, polyploidy induction

and micronucleus evaluation. There is also experimental evidence that spindle poisons can be detected in MLA. The preferred in vivo cytogenetic test under Option 2 is the micronucleus assay, not a chromosome aberration assay, to include more direct capability for detection of chromosome loss (potential for aneuploidy).

The suggested standard set of tests does not imply that other genotoxicity tests are generally considered inadequate or inappropriate. Additional tests can be used for further investigation of genotoxicity test results obtained in the standard battery. Alternative species, including non-rodents, can also be used if indicated, and if sufficiently validated.

Under conditions in which one or more tests in the standard battery cannot be employed for technical reasons, alternative validated tests can serve as substitutes provided sufficient scientific justification is given.

Chapters (2-5) of the ICH S2(R1) Guideline contain also detailed information and modifications to the test battery and specific recommendations to the *in vitro* and *in vivo* studies and on evaluation of test results and on follow-up test strategies.

6. Veterinary Drugs

A battery of three tests is recommended in the VICH GL23 Guideline (October 2012, under revision) for use as a screen of veterinary drugs for genotoxicity *[Chapter 2]*:

i. A test for gene mutation in bacteria.
ii. A cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma tk gene mutation assay.
iii. An *in vivo* test for chromosomal effects using rodent haematopoietic cells.

Modifications can be applied and modified protocols should be used where it is evident that standard conditions will give a false negative result.

7. Other relevant documents

7.1 EFSA Opinion on genotoxicity testing strategies

This is an EFSA Scientific Committee Review of the current state-of-the-science on genotoxicity testing providing a commentary and recommendations on genotoxicity testing strategies (EFSA, 2011). A step-wise approach is recommended for the generation and evaluation of data on genotoxic potential, beginning with a basic battery of *in vitro* tests, comprising a bacterial reverse mutation assay and an *in vitro* micronucleus assay. It is suggested to consider whether specific features of the test substance might require substitution of one or more of the recommended *in vitro* tests by other *in vitro* or *in vivo* tests in the basic battery. In the event of negative *in vitro* results, it can be concluded that the substance has no genotoxic potential. In case of inconclusive, contradictory or equivocal results, it may be appropriate to conduct further testing *in vitro*. In case of positive *in vitro* results, review of the available relevant data on the test substance and, where necessary, an appropriate *in vivo* study to assess whether the genotoxic potential observed *in vitro* is expressed *in vivo* is recommended. The approach to *in vivo* testing should be also step-wise. The combination of assessing different endpoints in different tissues in the same animal *in vivo* should also be considered.

7.2 UK Committee on Mutagenicity of Chemicals in Food (COM) Guidance

The COM Guidance provides a strategy for testing chemical substances where no genotoxicity data are available (COM, 2011).

- *Stage 0*: Preliminary considerations prior to testing. Analysis of Physico-chemical and Toxicological Properties; (Q)SAR models for prediction of mutagenic activity; Screening tools ie. *in silico* approach].
- *Stage 1*: *In vitro* genotoxicity testing. The strategy includes using appropriate tests to gain an insight into the nature of the genotoxic effects of a test substance and also to ***avoid false positive results***. It comprises a two-test core system (namely an Ames test and *in vitro* micronucleus test) with the objective of assessing mutagenic potential by investigating three different end points (gene mutation, structural chromosomal damage and changes in chromosome number).
- *Stage 2*: *In vivo* genotoxicity testing. The *in vivo* genotoxicity testing strategy has to be designed on a case-by case basis and can be used to address aspects of *in vivo* mutagenicity.

7.3 Review on Alternative (non-animal) methods for cosmetics testing (Adler et al., 2011)

With regard to the 7th Amendment to the EU Cosmetics Directive to prohibit animal-tested cosmetics on the market from 2013, the European Commission invited stakeholder bodies (industry, non-governmental organisations, EU Member States, and the

Commission's Scientific Committee on Consumer Safety) to identify scientific experts in five toxicological areas, i.e. toxicokinetics, repeated dose toxicity, carcinogenicity, skin sensitisation, and reproductive toxicity for which the Directive foresees that the 2013 deadline could be further extended in case alternative and validated methods would not be available in time. The selected experts were asked to analyse the status and prospects of alternative methods and to provide a scientifically sound estimate of the time necessary to achieve full replacement of animal testing.

In relation to the carcinogenicity endpoint, the experts evidenced impediments for a full replacement of animal testing in due time.

They stated that the carcinogenic potential of a chemical substance is far to be fully determined and/or mimicked by the use of non-animal testing because of the complexity of the carcinogenesis process, the multi-stage nature of its evolution and complex biological interactions. The 2-year cancer bioassay in rodents is widely regarded as the gold standard to evaluate cancer hazard and potency; however this test is rarely done on cosmetics ingredients. Instead a combination of shorter-term studies has been used, including *in vitro* and *in vivo* genotoxicity assays to assess genotoxic potential and repeated-dose (typically 90-day) toxicity studies to assess the risk of non genotoxic chemicals. Therefore, their conclusion was that the animal testing ban under the 7th Amendment to the Cosmetics Directive will have a strong impact on the ability to evaluate and conduct a quantitative risk assessment for carcinogenic potential of cosmetics ingredients. This impact is not only due to the ban on the cancer bioassay itself, but mainly to the ban of *in vivo* genotoxicity testing, any repeated-dose toxicity testing and other tests such as *in vivo* toxicokinetics studies and *in vivo* mechanistic assays.

Although several *in vitro* short-term tests at different stages of development and acceptance are available, at the current status these will not be sufficient to fully replace the animal tests needed to confirm the safety of cosmetics ingredients. However, for some chemical classes, the available methods might be sufficient to rule out carcinogenic potential in a weight of evidence approach. Taking into consideration the state of the art of non-animal methods, the experts were unable to suggest a timeline for full replacement of animal tests currently needed to fully evaluate carcinogenic risk of chemicals.

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Acknowledgements

This Report was prepared by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), part of the Institute for Health and Consumer Protection (IHCP), Directorate-General Joint Research Centre (DG JRC) of the European Commission.

The Report benefitted from comments and suggestions received from members of PARERE (EURL ECVAM's advisory body for Preliminary Assessment of Regulatory Relevance that brings together representatives of Member State regulatory bodies as well as EU agencies including ECHA, EFSA and EMA), and ESTAF (EURL ECVAM's Stakeholder Forum). Input was also provided by partner organisations of EURL ECVAM in the framework of the International Collaboration on Alternative Test Methods (ICATM).

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European Commission
EUR 26375 – Joint Research Centre – Institute for Health and Consumer Protection

Title: EURL ECVAM Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing

Author(s): Raffaella Corvi, Federica Madia, Andrew Worth and Maurice Whelan

Luxembourg: Publications Office of the European Union

2013 – 48 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1018-5593 (print) ISSN 1831-9424 (online)

ISBN 978-92-79-34844-0 (pdf)
ISBN 978-92-79-34845-7 (print)

doi: 10.2788/43865 (online)

Abstract

The assessment of genotoxicity represents an important component of the safety assessment of all types of substances. Although several in vitro tests are available at different stages of development and acceptance, they cannot at present be considered to fully replace animal tests needed to evaluate the safety of substances. Based on an analysis of regulatory requirements for this endpoint within different pieces of EU legislation, EURL ECVAM proposes a pragmatic approach to improve the traditional genotoxicity testing paradigm that offers solutions in both the short- and medium-term and that draws on the considerable experience of 40 years of regulatory toxicology testing in this area. EURL ECVAM considers that efforts should be directed towards the overall improvement of the current testing strategy for better hazard and risk assessment approaches, which either avoids or minimises the use of animals, whilst satisfying regulatory information requirements, irrespective of regulatory context. Several opportunities for the improvement of the testing strategy have been identified which aim to i) enhance the performance of the in vitro testing battery so that fewer in vivo follow-up tests are necessary and ii) guide more intelligent in vivo follow-up testing to reduce unnecessary use of animals. The implementation of this strategic plan will rely on the cooperation of EURL ECVAM with other existing initiatives and the coordinated contribution from various stakeholders.

LB-NA-26375-EN-N

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Publications Office

ISBN 978-92-79-34844-0

9 789279 348440