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Guidance on Information Requirements and Chemical Safety Assessment

Extract from

Chapter R.7a: Endpoint specific guidance

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Guidance on Information Requirements and Chemical Safety Assessment
Chapter R.7a: Endpoint specific guidance

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Preface

This document describes the information requirements under REACH with regard to substance properties, exposure, uses and risk management measures, and the chemical safety assessment. It is part of a series of guidance documents that are aimed to help all stakeholders with their preparation for fulfilling their obligations under the REACH Regulation. These documents cover detailed guidance for a range of essential REACH processes as well as for some specific scientific and/or technical methods that industry or authorities need to make use of under REACH.

The guidance documents were drafted and discussed within the REACH Implementation Projects (RIPs) led by the European Commission services, involving stakeholders from Member States, industry and non-governmental organisations. After acceptance by the Member States Competent Authorities the guidance documents had been handed over to ECHA for publication and further maintenance. Any updates of the guidance are drafted by ECHA and are then subject to a consultation procedure, involving stakeholders from Member States, industry and non-governmental organisations. For details of the consultation procedure, please see:

https://echa.europa.eu/documents/10162/17207/pro-0011_consultation_procedure_for_guidance_en.pdf

The guidance documents can be obtained *via* the website of the European Chemicals Agency:

https://echa.europa.eu/guidance-documents/guidance-on-reach

This document relates to the REACH Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006[1].

[1] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (OJ L 396 of 30 December 2006, p. 1; corrected by OJ L 136, 29.5.2007, p. 3).

NOTE

Please note that the present document is a proposed amendment to **specific sections only** of the *Chapter R.7a* of the *Guidance on IR&CSA*.

This document was prepared by the ECHA Secretariat for the purpose of this consultation and includes only the parts open for the current consultation, i.e. **sections R.7.7.1 to R.7.7.7 related to Mutagenicity**.

The current version of the full guidance document (version before the proposed amendment) is available on the ECHA website at:

https://echa.europa.eu/documents/10162/17224/information_requirements_r7a_en.pdf/e4a2a18f-a2bd-4a04-ac6d-0ea425b2567f?t=1500286622893

The numbering and headings of the sub-sections that are displayed in the document for consultation correspond to those used in the currently published guidance document; this will enable the comparison of the draft revised sub-sections with the current text if necessary.

After conclusion of the consultation and before final publication, the updated sections will be implemented in the full document.

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R.7.7 Mutagenicity and carcinogenicity

R.7.7.1 Mutagenicity

R.7.7.1.1 Definition of mutagenicity

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes may involve a single gene or gene segment, a block of genes or chromosomes. Several mutagenicity endpoints (i.e. gene mutation, clastogenicity and aneuploidy) can be distinguished. The term gene mutation refers to permanent changes in the base sequences of a certain gene. 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 rearrangements of chromosome segments. Aneugenicity (aneuploidy induction) refers to the effects of agents that give rise to a change (gain or loss) in chromosome number in cells. An aneugen can cause loss or gain of chromosomes resulting in cells that have not an exact multiple of the haploid number. For example, three number 21 chromosomes or trisomy 21 (characteristic of Down syndrome) is a form of aneuploidy.

Genotoxicity is a broader term and refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity. Thus, tests for genotoxicity include tests which provide an indication of induced damage to DNA (but not direct evidence of mutation) *via* effects such as DNA strandbreaks, unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE), DNA adduct formation or mitotic recombination, as well as tests for mutagenicity.

The chemical and structural complexity of the chromosomal DNA and associated proteins of mammalian cells, and the multiplicity of ways in which changes to the genetic material can be affected make it difficult to give more precise, discrete definitions.

In the risk assessment of substances, it is necessary to address the potential effect of *mutagenicity*. It can be expected that some of the available data will have been derived from tests conducted to investigate potentially harmful effects on genetic material (*genotoxicity*). Hence, both the terms *mutagenicity* and *genotoxicity* are used in this document.

R.7.7.1.2 Objective of mutagenicity assessment

The aim of testing for genotoxicity is to assess the potential of substances to induce genotoxic effects, which may lead to cancer or cause heritable damage in humans. Genotoxicity data are used in risk characterisation and classification of substances. Genotoxicity data are useful for the determination of the general mode of action of a substance (*i.e.* type(s) of genotoxic damage induced) and can provide some indication on the dose (concentration)-response relationship and on whether the observed effect(s) can be reasonably assumed to have a threshold or not. Genotoxicity data can thus be informative on the appropriate approach to use for risk assessment. Expert judgement is necessary at each stage of the testing strategy to decide on the relevance of a result based on the data available for each mutagenicity endpoint.

Alterations to the genetic material of cells may occur spontaneously endogenously or be induced as a result of exposure to ionising or ultraviolet radiation, or genotoxic substances. In principle, human exposure to substances that are mutagens may result in increased frequencies of mutations above the background.

Mutations in somatic cells may be lethal for the cells or may be transferred to daughter cells with potentially deleterious consequences for the affected organism (*e.g.* cancer may be induced when mutations occur in proto-oncogenes, tumour suppressor genes and/or DNA repair genes).

Heritable damage to the offspring, and possibly to subsequent generations, may follow if mutations are induced in germ cells of parents exposed to substances that are mutagens. To date, all known germ cell mutagens are also mutagenic in somatic cells *in vivo.* Substances that are mutagenic in somatic cells may produce heritable effects if they, or their active metabolites, have the ability to interact with the genetic material of germ cells. Conversely, substances that do not induce mutations in somatic cells *in vivo* would not be expected to be germ cell mutagens.

There is considerable evidence of a positive correlation between the mutagenicity of substances *in vivo* and their carcinogenicity in long-term studies with animals. Genotoxic carcinogens are substances for which the most plausible mechanism of carcinogenic action involves genotoxicity.

R.7.7.2 Information requirements on mutagenicity

The information requirements on mutagenicity are described by REACH Annexes VI-X, that specify the information that must be submitted for registration and evaluation purposes. The information is thus required for substances produced or imported in quantities of >1 t/y (tons per annum). When a higher tonnage level is reached, the requirements of the corresponding Annex have to be considered. However, factors including not only production volume but also pre-existing toxicity data, information about the identified use of the substance and exposure of humans to the substance will influence the precise information requirements. The REACH Annexes must thus be considered as a whole, and in conjunction with the overall requirements of registration, evaluation and the duty of care.

Column 1 of REACH Annexes VII-X defines the standard information requirements for substances produced or imported in quantities of >1 t/y, >10 t/y, >100 t/y, and >1000 t/y, respectively.

Column 2 of REACH Annexes VII-X lists specific rules according to which the required standard information may be omitted, replaced by other information, provided at a different stage or adapted in another way, or may trigger further information requirement(s). If the conditions are met under which column 2 of these Annexes allows adaptations, the fact and the reasons for each adaptation should be clearly indicated in the registration dossier.

The standard information requirements for mutagenicity and the specific rules for adaptation of these requirements are presented in Table R.7.7–1.

Table R.7.7–1 REACH information requirements for mutagenicity

COLUMN 1 STANDARD INFORMATION REQUIRED	COLUMN 2 SPECIFIC RULES FOR ADAPTATION FROM COLUMN 1
Annex VII: 8.4. Mutagenicity	8.4. In case of a positive result in the *in vitro* gene mutation study in bacteria referred to in point 8.4.1 of this Annex, which gives rise to concern, the registrant shall perform an *in vitro* study referred to in Annex VIII, point 8.4.2. Based on the positive result of any of those *in vitro* genotoxicity studies, the registrant shall propose, or the Agency may require, an appropriate *in vivo* study referred to in Annex IX, point 8.4.4. The *in vivo* study shall address the chromosomal aberration concern or the gene mutation concern or both, as appropriate. The *in vitro* gene mutation study in bacteria does not need to be conducted if this test is not applicable for the substance. In this case, the registrant shall provide a justification and perform an *in vitro* study referred to in Annex VIII, point 8.4.3. In case of a positive result in that study the registrant shall perform an *in vitro* cytogenicity study referred to in Annex VIII, point 8.4.2. Based on the positive result in any of those *in vitro* genotoxicity studies, or in case one of the Annex VIII *in vitro* tests is not applicable for the substance, the registrant shall propose, or the Agency may require, an appropriate *in vivo* study referred to in Annex IX, point 8.4.4. The *in vivo* study shall address the chromosomal aberration concern or the gene mutation concern or both, as appropriate. The *in vitro* gene mutation study in bacteria referred to in point 8.4.1 and follow-up testing do not need to be conducted in any of the following cases: — the substance is known to cause germ cell mutagenicity, meeting the criteria for classification in the hazard class germ cell mutagenicity category 1A or 1B, and appropriate risk management measures are implemented, — the substance is known to be a genotoxic carcinogen, meeting the criteria for classification both in the hazard class germ cell mutagenicity category 1A, 1B or 2 and in the hazard class carcinogenicity category 1A or 1B, and appropriate risk management measures are implemented.’;
8.4.1. *In vitro* gene mutation study in bacteria.	8.4.1. The *in vitro* gene mutation study in bacteria does not need to be conducted for nanoforms where it is not appropriate. In such case, an *in vitro* study referred to in Annex VIII, point 8.4.3, shall be provided.’;

Annex VIII: 8.4. Mutagenicity 8.4.2. *In vitro* mammalian chromosomal aberration study or *in vitro* mammalian micronucleus study. 8.4.3. *In vitro* gene mutation study in mammalian cells, if a negative result in Annex VII, Section 8.4.1. and Annex VIII, Section 8.4.2.	8.4. The studies referred to in points 8.4.2 and 8.4.3 do not need to be conducted in any of the following cases: — adequate data from the corresponding *in vivo* study, (namely *in vivo* chromosomal aberration (or micronucleus) study regarding point 8.4.2 or *in vivo* mammalian gene mutation study regarding point 8.4.3), are available, — the substance is known to cause germ cell mutagenicity, meeting the criteria for classification as germ cell mutagen category 1A or 1B, and appropriate risk management measures are implemented, — the substance is known to be a genotoxic carcinogen, meeting the criteria for classification both in the hazard class germ cell mutagenicity category 1A, 1B or 2 and in the hazard class carcinogenicity category 1A or 1B, and appropriate risk management measures are implemented. In case of a positive result in any of the *in vitro* genotoxicity studies referred to in Annex VII or this Annex, which gives rise to concern, the registrant shall propose, or the Agency may require, an appropriate *in vivo* study referred to in Annex IX, point 8.4. The *in vivo* study shall address the chromosomal aberration concern or the gene mutation concern or both as appropriate. In case an *in vitro* mutagenicity study referred to in points 8.4.2 or 8.4.3 is not applicable for the substance, the registrant shall provide a justification and shall propose or the Agency may require an appropriate *in vivo* study referred to in Annex IX, point 8.4.4. The *in vivo* study shall address the chromosomal aberration concern or the gene mutation concern or both as appropriate.’;

Annex IX: 8.4. Mutagenicity	8.4. The studies referred to in points 8.4.4 and 8.4.5 do not need to be conducted in any of the following cases: — the substance is known to cause germ cell mutagenicity, meeting the criteria for classification in the hazard class germ cell mutagenicity category 1A or 1B, and appropriate risk management measures are implemented, — the substance is known to be a genotoxic carcinogen, meeting the criteria for classification both in the hazard class germ cell mutagenicity category 1A, 1B or 2 and in the hazard class carcinogenicity category 1A or 1B, and appropriate risk management measures are implemented.‘;
8.4.4. An appropriate *in vivo* mammalian somatic cell genotoxicity study, if there is a positive result in any of the *in vitro* genotoxicity studies referred to in Annex VII or Annex VIII, which gives rise to concern. The *in vivo* mammalian somatic cell genotoxicity study shall address the chromosomal aberration concern or the gene mutation concern or both, as appropriate.	8.4.4. The *in vivo* mammalian somatic cell genotoxicity study does not need to be conducted if there are adequate results available from an appropriate *in vivo* mammalian somatic cell genotoxicity study.
8.4.5. An appropriate *in vivo* mammalian germ cell genotoxicity study, if there is a positive result in an available *in vivo* mammalian somatic cell genotoxicity study, which gives rise to concern. The *in vivo* mammalian germ cell genotoxicity study shall address the chromosomal aberration concern or the gene mutation concern or both, as appropriate.	8.4.5. The study does not need to be conducted if there is clear evidence that neither the substance nor its metabolites reach the germ cells.‘;

Annex X:	
8.4. Mutagenicity	8.4. The studies referred to in points 8.4.6 and 8.4.7 do not need to be conducted in any of the following cases: — the substance is known to cause germ cell mutagenicity, meeting the criteria for classification in the hazard class germ cell mutagenicity category 1A or 1B, and appropriate risk management measures are implemented, — the substance is known to be a genotoxic carcinogen, meeting the criteria for classification both in the hazard class germ cell mutagenicity category 1A or 1B or 2 and in the hazard class carcinogenicity category 1A or 1B, and appropriate risk management measures are implemented.’;
8.4.6. A second *in vivo* mammalian somatic cell genotoxicity study, if there is a positive result in any of the *in vitro* genotoxicity studies referred to in Annex VII or Annex VIII, which gives rise to both chromosomal aberration concern and gene mutation concern. The second study shall address chromosomal aberration or gene mutation, as appropriate, which has not been addressed by the first *in vivo* mammalian somatic cell genotoxicity study.	
8.4.7. A second *in vivo* mammalian germ cell genotoxicity study, if there is a positive result in *in vivo* mammalian somatic cell genotoxicity studies, which gives rise to both chromosomal aberration concern and gene mutation concern. The second study shall address the chromosomal aberration or gene mutation, as appropriate, which has not been addressed by the first *in vivo* mammalian germ cell genotoxicity study.	8.4.7. The study does not need to be conducted if there is clear evidence that neither the substance nor its metabolites reach the germ cells.’;

In addition to these specific rules, the required standard information set may be adapted according to the general rules contained in REACH Annex XI. In this case as well, the fact and the reasons for each adaptation should be clearly indicated in the registration dossier.

In some cases, the rules set out in Annex VII to X may require certain tests to be undertaken earlier than or in addition to the tonnage-triggered requirements. Registrants should note that a testing proposal must be submitted for a test mentioned in Annex IX or X, independently from the registered tonnage. Following examination of such a testing proposal ECHA has to approve the test in its evaluation decision before it can be undertaken. See Section R.7.7.6 of this Guidance for further guidance on testing requirements.

R.7.7.3 Information sources on mutagenicity

To be able to evaluate the mutagenic potential of a substance in a comprehensive way, information is required on its ability to induce gene mutations, structural chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity). Many test methods are available by which such information can be obtained. Non-testing methods, such as SAR, QSAR and read-across approaches, may also provide information on the mutagenic potential of a substance.

Typically, *in vitro* tests are performed with cultured bacterial cells, human or other mammalian cells. The applicability of these tests will vary with different classes of substances and can guide the selection of the most appropriate test systems to be used. In order to detect mutagenic effects also of substances that need to be metabolically activated to become mutagenic, an exogenous metabolic activation system is usually added in *in vitro* test systems. For this purpose, the post-mitochondrial 9000 x *g* supernatant (S9 fraction) of whole liver tissue homogenate, containing a high concentration of metabolising enzymes and extracted from animals (usually rats) that have been induced to raise the oxidative cytochrome P450 levels, is most commonly employed.

When information is required on the mutagenic potential of a substance *in vivo,* several test methods are available. In *in vivo* tests, metabolism of the substance and its toxicokinetic properties can determine the genotoxic response of the test animal. It should be noted that species-specific differences in metabolism and toxicokinetics are known. Therefore, different genotoxic responses may be obtained using different species. Some *in vivo* genotoxicity tests, such as the Transgenic rodent (TGR) somatic and germ cell gene mutation assays and the *in vivo* comet assay, employ methods by which any tissue (containing nucleated cells) of an animal can in theory be examined for effects on the genetic material. This gives the possibility to examine distant target tissues (including germ cells) and site-of-contact tissues (*i.e.* skin, epithelium of the respiratory or gastro-intestinal tract). However, differences can exist regarding the number and type of tissues for which the use of a specific test has been scientifically validated. For instance, the TGR assays can be used to examine germ cells whereas the comet assay as described in the corresponding OECD test guideline (TG) 489 is, at present, not recommended for that purpose.

Some test methods, but not all, have an officially adopted EU and/or OECD TG. In cases where no adopted EU or OECD TG is available for a test method, rigorous and robust protocols should be followed, such as those defined by internationally recognised groups of experts like the International Workshop on Genotoxicity Testing (IWGT), under the umbrella of the International Association of Environmental Mutagen Societies. Furthermore, modifications to OECD TGs have been developed for some classes of substances and may enhance the accuracy of test results. Use of such modified protocols is a matter of expert judgement and will vary as a function of the chemical and physical properties of the substance to be evaluated. Similarly, the use of standard test methods for the testing of tissue(s) not covered by those standard test methods should be scientifically justified and validity of the results will depend on the appropriateness of the acceptability criteria, which should have been specifically developed for this (these) tissue(s) based on sufficient experience and historical data.

R.7.7.3.1 Non-human data on mutagenicity

Non-testing data on mutagenicity

Non-test information about the mutagenicity of a substance can be derived in a variety of ways, ranging from simple inspection of the chemical structure through various read-across techniques, the use of expert systems, metabolic simulators, to *global* or *local* (Q)SARs. The usefulness of such techniques varies with the amount and nature of information available, as well as with the specific regulatory questions under consideration.

Weight of Evidence

Regarding substances for which testing data exist, non-test information can be used in the *Weight of Evidence* approach, to help confirm results obtained in specific tests, or to help develop a better understanding of mutagenicity mechanisms. The information may be useful in deciding if, or what, additional testing is required. At the other extreme, where no testing data are available, similar alternative sources of information may assist in setting test priorities. In cases where no testing is likely to be done (e.g. <1 t/y), non-testing data may be the only options available to establish a hazard profile.

Read-across

Grouping of substances and read-across can also be used to predict the mutagenic properties of the 'target' substance from the data available on analogue 'source' substances. Read-across and chemical categories are described in Section R.6.2 of the *Guidance on IR&CSA*, *Chapter R.6*. The scientific basis for building grouping arguments and read-across cases were revisited in the second version of the OECD Guidance Document on grouping of chemicals (OECD, 2014). More detailed advice on the scientific assessment of read-across under REACH can be found in ECHA's Read-Across Assessment Framework (RAAF) document (see https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across).

(Q)SAR prediction models for mutagenicity

There are hundreds of (Q)SAR models available in the literature (Honma, 2020). There are *local* (Q)SARs, for relatively small sets of congeneric substances, and global models for a wide variety of non-congeneric substances. Global models may constitute valuable predictive tools for estimating a number of mutagenic/genotoxic endpoints, if essential features of the information domain are clearly represented. However, quality of reporting varies from model to model and predictivity must be assessed case-by-case on the basis of clear documentation. Use of harmonised templates, such as the QSAR Model Reporting Format (QMRF) and the QSAR Prediction Reporting Format (QPRF) can help ensure consistency in summarising and reporting key information on (Q)SAR models and substance-specific predictions generated by (Q)SAR models.

Generally, (Q)SAR models (local or global) that contain putative mechanistic descriptors are preferred. However, many models use purely structural descriptors. While such models may be highly predictive, they rely on statistical methods and the toxicological significance of the descriptors may be obscure. QSAR modelling could be done even without descriptors (Hung and Gini, 2021).

Global (Q)SARs are usually implemented in computer programs and may comprise a set of local models; these global models first categorise the input molecule into the chemical domain it belongs to, and then apply the corresponding local prediction model. These are known as expert systems. Other global models apply the same mathematical algorithm on all input molecules without prior separation. These are known as statistical models. The concept of applicability domain is important and the endpoints for substances inside the applicability domains of the models are better predicted than for substances falling outside.

Many global models for mutagenicity are commercial and some of the suppliers of these global models consider the data in their modelling sets to be proprietary. Proprietary means that the training set data used to develop the (Q)SAR model is hidden from the user. In other cases, it means that it may not be distributed beyond use by regulatory authorities. The models do not always equal the software incorporating them, and the software often has flexible options for expert uses. Thus, the level of information available, from both (Q)SAR models and compiled databases, should be adequate for the intended purpose.

A list of the available (free and commercial) predictive software for ecotoxicological, toxicological and environmental endpoints, including mutagenicity models, has been compiled within the frame of an EU project (https://www.life-concertreach.eu/results/results-gateway/). This website contains information for about 450 models, sorted by endpoint. The JRC website hosts the JRC (Q)SAR Model Inventory, which is an inventory of information of (Q)SAR models that have been submitted to the JRC (https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/QSARDB/LATEST/qsardb.html). This inventory contains a list of 154 models currently, out of which 21 cover mutagenicity endpoints).

The *in vitro* bacterial mutagenicity (Ames) test is the most common predicted genotoxicity endpoint for global models. For pesticides which belong to different regulatory domain but still within the chemical domain, (Q)SAR models (from commercial and free software) generated predictions that result in average 80% accuracy. This accuracy is comparable to the reported experimental variability of the Ames test results (80–84% repeatability from laboratory to laboratory). The Ames test appears to be well predicted while reliability of the (Q)SAR models for other genotoxicity assays/endpoints is still quite far from optimal (Benigni et al, 2019). As a general trend, the combination of QSARs increases sensitivity, but at the expense of specificity.

There are models for many other mutagenicity endpoints. For example, the Danish EPA and the Danish QSAR group at DTU Food (National Food Institute at the Technical University of Denmark) have developed a (Q)SAR database that contains predictions from a number of mutagenicity models. In addition to assorted Ames models, the database provides predictions of the following *in vitro* endpoints: chromosomal aberrations (CHO and CHL cells), mouse lymphoma/*tk*, CHO/hprt gene-mutation assays and UDS (rat hepatocytes); and the following *in vivo* endpoints: *Drosophila* SLRL, mouse micronucleus, rodent dominant lethal, mouse SCE in bone marrow and mouse comet assay data. The Danish QSAR database is a repository of model estimates for more than 600,000 substances and is considered as a good screening tool. All organic single constituent substances that were pre-registered or registered under REACH (around 80,000) are included in the structure set. In addition, chemical structures from other relevant databases are included, leading to the new structure set of more than 600,000 unique chemical structures. When possible, the endpoints have been modelled in the three software systems Leadscope, CASE Ultra and SciQSAR. All DTU in-house models and a number of commercial models from MultiCASE® have been modelled in two or three systems. For the set structure, predictions are provided in the different systems separately and as an overall battery prediction. A user manual with information on the individual models including training set information and validation results is available at the website. Predictions from a number of OECD QSAR Toolbox profilers have also been included as supporting information to the QSAR predictions. The database also includes predictions from other software (e.g. VEGA). The Danish QSAR database is freely accessible *via* https://qsar.food.dtu.dk/ and is also integrated into the OECD QSAR Toolbox.

Another example of a database with predictions on mutagenicity is the Enhanced NCI Database Browser (https://cactus.nci.nih.gov) sponsored by the U.S. National Cancer Institute. It contains predictions for over 250,000 substances for mutagenicity as well as other non-mutagenic endpoints, some of which may provide valuable mechanistic information (for example alkylating ability or microtubule formation inhibition). It is also searchable by a wide range of parameters and structure combinations.

Neither of these two examples is perfect, but they illustrate a trend towards predictions of multiple endpoints and may assist those making *Weight of Evidence* decisions regarding the mutagenic potential of untested substances.

For mutagenicity predictions, the potential of the substances to generate metabolites of concern should also be considered. Some models for genetic toxicity include metabolic simulator and prediction of metabolites. In addition, separate *in silico* approaches to predict the likely metabolites based on molecular structure are available. Reliability of simulated metabolism is discussed in Dermen *et al.* (2022). Metabolism can explain some differences

between *in vitro* and *in vivo* genotoxicity (Petkov *et al.*, 2022). A new QSAR modelling concept was introduced where the potency is related to the amount of DNA adducts as an element of metabolism prediction.

OECD QSAR Toolbox

The OECD QSAR Toolbox (https://qsartoolbox.org/) is a freely available software developed by ECHA and OECD that supports reproducible and transparent chemical hazard assessment. It offers functionalities for retrieving experimental data, simulating metabolism and profiling properties of chemicals. These pieces of information and tools can also be used to identify structurally and mechanistically defined analogues and chemical categories, which can serve as sources for read-across and trend analysis.

Concerning mutagenicity, the OECD QSAR Toolbox covers the *in vitro* gene mutation (Ames test), *in vitro* chromosomal aberration, *in vivo* chromosomal aberration (micronucleus test), and genotoxic carcinogenicity endpoints. The OECD QSAR Toolbox includes a number of databases with relevant experimental data (ECHA REACH, Food TOX Hazard EFSA, Bacterial mutagenicity ISSSTY, Genotoxicity and Carcinogenicity ECVAM, Genotoxicity OASIS, Genotoxicity pesticides EFSA, Micronucleus ISSMIC, Micronucleus OASIS, Toxicity Japan MHLW, and Transgenic Rodent Database). It also offers profilers to predict mode of actions that are relevant for mutagenicity, such as the profilers DNA binding by OASIS and OECD, DNA alerts for AMES, CA and MNT by OASIS, and *in vitro* and *in vivo* mutagenicity alerts by ISS. Data and profilers can be used in combination to identify mechanistically and structurally relevant analogues for read-across predictions. The OECD QSAR Toolbox includes a number of QSAR models to predict mutagenicity endpoints from the Danish QSAR database. Any prediction obtained from the OECD QSAR Toolbox has to be critically assessed before use.

A list of existing software for mutagenicity predictions is presented in Table R.7.7–2 List of software that might be used to predict different mutagenicity endpoints.

Table R.7.7–2 List of software that might be used to predict different mutagenicity endpoints

Endpoint	Software	Models	Free or commercial
Mutagenicity *in vitro* (Ames test)	Danish QSAR Database (DTU)	Models for Ames test	Free
	OECD QSAR Toolbox (LMC)	Several profilers with alerts, DNA alerts for Ames	Free
	T.E.S.T (US EPA)	Mutagenicity	Free
	ToxTree (JRC)	*in vitro* mutagenicity alerts by ISS	Free
	VEGA (IRFMN)	CAESAR, ArPy/IRFMN, ISS and KNN/Read-across models	Free
	ACD Percepta	Genotoxicity module	Commercial
	CASE Ultra (MultiCASE)	Bacterial mutagenicity model bundle	Commercial
	DEREK and SARA (LHASA)	Mutagenicity *in vitro*	Commercial

	Leadscope	Genetox Expert Alerts Suite	Commercial
	TIMES (LMC)	Genotoxicity *in vitro*/ Ames S9 activated	Commercial
	ChemTunes (Molecular Networks and Altamira)	ChemTunes, ToxGPS Ames (enhanced)	Commercial
Mutagenicity - other endpoints	Danish QSAR Database (DTU)	Models for mammalian cells *in vitro*, *in vivo* genotoxicity	Free
	OECD QSAR Toolbox (LMC)	Several profilers with alerts, DNA alerts for CA and MNT, Protein binding alerts for CA	Free
	CASE Ultra (MultiCASE)	Micronucleus, chromosomal aberrations, sister chromatid exchange models	Commercial
	DEREK and SARA (LHASA)	Chromosome damage *in vitro* and *in vivo*, mutagenicity *in vivo*	Commercial
	Leadscope	Non-human genetic toxicity	Commercial
	TIMES (LMC)	Chromosomal aberration S9 activated, mouse lymphoma S9 activated	Commercial

Testing data on mutagenicity

Standard test methods appropriate for investigation of mutagenicity are listed in Table R.7.7–3, Table R.7.7–4 and Table R.7.7–5. A general update of the OECD TGs for genetic toxicity testing was done in 2014-2015 and an overview of the changes is provided in the OECD Series on Testing and Assessment No. 238 - 2nd edition (OECD, 2017). Even if some of these TGs have been further updated and one new TG has been adopted since publication of this document, it still contains relevant background information and addresses important aspects related to the selection and application of the assays for genetic toxicology. For further information and access to the latest versions of these OECD TGs, please see: https://www.oecd.org/env/ehs/testing/.

In vitro data

Table R.7.7–3 *In vitro* test methods

Test method	GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD	OECD/EU guideline[a]
Bacterial reverse mutation test	Gene mutations / The test uses amino-acid requiring strains of bacteria to detect (reverse) gene mutations (point mutations and frameshifts).	OECD: 471 EU: B.13/14
In vitro mammalian cell gene mutation tests – HPRT and XPRT genes	Gene mutations / The test identifies substances that induce gene mutations in the *hprt* and *xprt* genes of established cell lines.	OECD: 476 EU: B.17
In vitro mammalian cell gene mutation tests – Thymidine kinase gene (Mouse lymphoma MLA and TK6 assays)	Gene mutations and structural chromosome aberrations / The test identifies substances that induce gene mutations in the *tk* gene of the L5178Y mouse lymphoma cell line and TK6 human lymphoblastoid cell line. If colonies in a *tk* mutation test are scored using the criteria of normal growth and slow growth colonies, gross structural chromosome aberrations (*i.e.* clastogenic effect) may be measured, since mutant cells that have suffered damage to both the *tk* gene and growth genes situated close to the *tk* gene have prolonged doubling times. The 'normal growing' and 'slow growing' mutants are recognised as 'large colony' and 'small colony' mutants in the MLA and as 'early appearing colony' mutants and 'late appearing colony' mutants in the TK6 assay.	OECD: 490 EU: B.17
In vitro mammalian cell micronucleus test	Structural and numerical chromosome aberrations / The test identifies substances that induce micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments or whole chromosomes, and the test thus has the potential to detect both clastogenic and aneugenic substances.	OECD: 487 EU: B.49
In vitro mammalian chromosome aberration test	Structural chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in cultured mammalian established cell lines, cell strains or primary cell cultures. An increase in polyploidy may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not recommended for that purpose.	OECD: 473 EU: B.10

[a] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

Accepted modifications to the standard test guidelines/methods have been developed to enhance test sensitivity to specific classes of substances and are described in the corresponding test guidelines. Expert judgement should be applied to determine whether any of these modifications are appropriate for a given substance being registered. For example, protocol modifications for the Ames test might be appropriate for substances such as gases, volatile liquids, azo-dyes, diazo compounds, glycosides, and petroleum oil derived products, which should be regarded as special cases.

In addition, some new *in vitro* test methods have been included in the OECD work programme with the aim to develop Detailed Review Papers (DRPs) on the test protocols and performances and potentially OECD TGs:

- Toxtracker uses stem cells and measures the expression of reporter genes involved in several genotoxic and non-genotoxic pathways linked to carcinogenicity.

- The *in vitro* γH2AX/phospho-Histone H3 assay is based on the phosphorylation states of specific histones (H2A and H3) used as early biomarkers of cellular response to DNA damage. It is claimed to have the ability to provide information on different mutagenicity modes of action, including a discrimination between clastogenic and aneugenic effects.

Animal data

- Somatic cells

Table R.7.7–4 *In vivo* test methods, somatic cells

Test method	GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD	EU/OECD guideline[a]
In vivo mammalian erythrocyte micronucleus test	Structural and numerical chromosome aberrations / The test identifies substances that cause micronuclei in erythroblasts sampled from bone marrow and/or peripheral blood cells of animals, usually rodents. These micronuclei originate from acentric fragments or whole chromosomes, and the test thus has the potential to detect both clastogenic and aneugenic substances.	OECD: 474 EU: B.12
In vivo mammalian bone marrow chromosome aberration test	Structural chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in the bone-marrow cells of animals, usually rodents. An increase in polyploidy may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not recommended for that purpose.	OECD: 475 EU: B.11
Transgenic rodent (TGR) somatic and germ cell gene mutation assays	Gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi- assay models) / Since the transgenes are transmitted by the germ cells, they are present in every cell. Therefore, gene mutations and/or chromosomal rearrangements can be detected in virtually all tissues of an animal, including target tissues and specific site of contact tissues.	OECD: 488 EU: B.58
In vivo mammalian alkaline comet assay	DNA strand breaks / The DNA strand breaks may result from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair. Therefore, the alkaline comet assay recognises primary DNA damage that would lead to gene mutations and/or chromosome aberrations, but will also detect DNA damage that may be effectively repaired or lead to cell death. The comet assay can be applied to almost every tissue of an animal from which single cell or nuclei suspensions can be made, including specific site of contact tissues.	OECD: 489 EU: B.62
Mammalian erythrocyte Pig-a gene mutation assay	Gene mutations / The erythrocyte Pig-a assay uses an endogenous mammalian gene, the phosphatidylinositol glycan class A gene (Pig-a), as a reporter of somatic-cell gene mutations in erythroid precursor cells, primarily found in the bone marrow. The test can identify substances that cause gene mutations in these precursor cells, which are reflected in erythrocytes sampled from peripheral blood cells of animals, usually rodents. The test can be conducted without euthanising the animals, which facilitates integration of the Pig-a assay into many *in vivo* rodent testing protocols.	OECD: 470 EU: none
Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*	DNA repair / The test identifies substances that induce DNA damage followed by DNA repair (measured as unscheduled "DNA" synthesis) in liver cells of animals, commonly rats. The test is usually based on the incorporation of tritium labelled thymidine into the DNA by repair synthesis after excision and removal of a stretch of DNA containing a region of damage. This test is no longer considered appropriate to generate new information under REACH[a].	OECD: 486 EU: obsoleted

[a] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

- Germ cells

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Table R.7.7–5 *In vivo* test methods, germ cells

Test method	GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD	EU/OECD guideline[a]
Mammalian spermatogonial chromosome aberration test	Structural chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in mammalian, usually rodent, spermatogonial cells and is, therefore, expected to be predictive of induction of heritable mutations in germ cells. An increase in polyploidy may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not routinely used for that purpose. Accordingly, this test guideline is not designed to measure numerical aberrations.	OECD: 483 EU: B.23
Transgenic rodent (TGR) somatic and germ cell gene mutation assays	Gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi- assay models) / Since the transgenes are transmitted by the germ cells, they are present in every cell. Therefore, gene mutations and/or chromosomal rearrangements can be detected in virtually all tissues of an animal including specific site of contact tissues and germ cells. Delayed sampling times may need to be considered in order to detect mutations in different stages of spermatogenesis.	OECD: 488 EU: B.58
Rodent dominant lethal test	Structural and numerical chromosome aberrations / The test identifies substances that induce dominant lethal effects causing embryonic or foetal death resulting from inherited dominant lethal mutations induced in germ cells of an exposed parent, usually the male. It is generally accepted that dominant lethal effects are due to structural and numerical chromosome aberrations. Rats or mice are recommended as the test species. This test is no longer considered appropriate to generate new information under REACH[a].	OECD: 478 EU: obsoleted

[a] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

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R.7.7.3.2 Human data on mutagenicity

Occasionally, studies of genotoxic effects in humans exposed by, for example, accident, occupation or participation in clinical studies (*e.g.* from case reports or epidemiological studies) may be available. Generally, cells circulating in blood are investigated for the occurrence of various types of genetic alterations.

R.7.7.4 Evaluation of available information on mutagenicity

Genotoxicity is a complex endpoint and requires evaluation by expert judgement. For both steps of the effects assessment, *i.e.* hazard identification and dose (concentration)-response (effect) assessment, it is very important to evaluate the data with regard to their adequacy and completeness. The evaluation of adequacy should address the reliability and relevance of the data in a way as outlined in the introductory section of this Guidance document. The completeness of the data refers to the conclusion on the comparison between the available adequate information and the information that is required under the REACH provisions for the applicable tonnage level of the substance. Such a conclusion relies on *Weight of Evidence* approaches, which categorise available information based on the methods used: *guideline tests*, *non-guideline tests*, and other types of information which may justify adaptation of the standard testing regime. Such a *Weight of Evidence* approach also includes an evaluation of the available data as a whole, *i.e.* both *over and across* toxicological endpoints (for example, consideration of existing carcinogenicity data, repeated dose toxicity data and genotoxicity data all together can help understand whether a substance could be a genotoxic or non-genotoxic carcinogen).

This approach provides a basis to decide whether further information is needed on endpoints for which specific data appear inadequate or not available, or whether the requirements are fulfilled.

R.7.7.4.1 Non-human data on mutagenicity

Non-testing data for mutagenicity

Read-across

The use of read-across for predicting the mutagenic properties of a target substance from the data from one or more source substance(s) must comply with the conditions set out in Annex XI, section 1.5 of the REACH Regulation. In particular, the read-across approach needs to be adequately and appropriately documented to support the read-across hypothesis and predictions.

The read-across assessment framework (RAAF) document, which is published on the ECHA website (https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across), describes a general framework and principles for the scientific assessment of the suitability of a read-across approach based on different scenarios. These scenarios are selected according to the type of read-across approach used (analogue or category approach), the basis for the read-across hypothesis ((Bio)transformation of the analogues into common compound(s) or different compounds have qualitatively similar properties) and whether quantitiative variations are expected in the predicted properties between the different analogues.

To justify the validity of a read-across approach, one important element of the RAAF is the need to specify why the commonalities between two or more analogue structures suggest a similar biological action. A justification also needs to be provided as to why structural dissimilarities are not expected to result in dissimilar biological action.

To assess the suitability of selected analogues as source substances, some general questions also need to be addressed:

- is the same endpoint considered?
- are there any additional functional groups or additional substituents that might

influence the reactivity and mutagenicity potential (applicability domain considerations)?
- are the physico-chemical parameters similar (applicability domain considerations)?
- are there impurities that influence the mutagenicity profile?
- is the likely chemical mechanism the same?

A read-across approach can also support a conclusion for a property within a weight-of-evidence approach.

(Q)SAR prediction models

When using (Q)SARs to predict a substance property, an assessment of both the model and the prediction is needed. Further Guidance on QSARs can be found in Section R.6.2 of the *Guidance on IR&CSA*, *Chapter R.6*, as well as in the OECD (Q)SAR assessment framework (QAF) that provides guidance and practical advice on how to assess the validity of models and their predictions (REF to be added upon publication in September). The QAF states that the validity of a model should be assessed according to the OECD validation principles for (Q)SARs (OECD, 2004; OECD, 2007), while the validity of predictions can be assessed against the newly established principles for the assessment of (Q)SAR predictions and results presented in the QAF Guidance. These new principles require that the input is correct, the substance is within the applicability domain of the model, the prediction is reliable, and the outcome is fit for the regulatory purpose.

For prediction of gene mutation in bacteria, it is important that all relevant strains of Ames test are addressed. Metabolic activation should be taken into account for adequacy and equivalence to tests (in order to make the predictions suitable to meet the information requirements for REACH). For statistical models, verification that the substance falls within the applicability domain and information on analogues supporting the predictions are important. Negative predictions from alert-based system like Derek Nexus, for example, can be considered only in the vicinity of very similar compounds that tested negative in the respective tests, supposed that all strains and metabolic activation are covered. These can be searched outside Derek Nexus (e.g. with Sarah Nexus, Vitic, or from another database source, such as the OECD QSAR Toolbox). The documentation, which Derek provides in the results window, however, is not sufficient to assess a prediction.

The Danish QSAR Database includes statistical models from SciQSAR, LeadScope and Case Ultra models for the Ames test. However, the documentation for the Danish QSAR Database does not always allow verification that the substance falls within the applicability domain and information from analogues substances, supporting the prediction. Information on models is however available from the website (https://qsardb.food.dtu.dk/)

If well-documented and applicable (Q)SAR data are available, they should be used to help reach the decision points described in the section below. In many cases the accuracy of such methods will be sufficient to help, or allow either a testing or a specific regulatory decision to be made. In other cases the uncertainty may be unacceptable due to the severe consequences of a possible error. This may be driven by many factors including high exposure potential or toxicological concerns.

The accuracy of available methods is best assessed by using substances that were not originally included in the training set of the models (the so-called external validation). There was an international challenge project for predicting Ames mutagenicity, results of which are described in Honma *et al*. (2019). Evaluating new data on mutagenicity can lead to expansion of the training sets (Amberg *et al*, 2019; Petkov et al, 2019a).

Documentation can include reference to a related substance or group of substances that leads to the conclusion of concern or lack of concern. This can either be presented according to a scientific logic (read-across justification) or sometimes as a mathematical relationship of

chemical similarity. It should be noted that when an *in silico* tool like the OECD QSAR Toolbox is used to find analogues and perform read-across, but not to make predictions based on (Q)SAR, this is considered as a read-across approach and the justification and predictions should comply with the conditions of a read-across adaptation.

The lack of mechanistic justification often limits the use of (Q)SAR predictions, especially for regulatory decisions. Workflows based on the combination of mechanistic (Q)SAR, read-across analysis and expert knowledge may be derived to allow users to make a transparent decision as to the final prediction based on *Weight of Evidence* (Petkov et al, 2019b). In case of consistent predictions, expert input may not be needed to make a final decision. Nonetheless, expert input may be useful to expand the set of read-across analogues from literature sources and/or to provide a rationale for the endpoint-specific similarity between source analogue(s) and the target substance. Advice on how to interpret some freely available models is provided by Mombelli *et al*. (2016). *Weight of Evidence* approach for Ames test predictions is also discussed in Mombelli *et al.* (2022).

Substances for which no test-data exist or for which testing is technically not possible represent a special case in which reliance on non-testing data may be absolute. Many factors will dictate the acceptability of non-testing methods in reaching a conclusion based on no tests at all. It may be discussed whether *Weight of Evidence* decisions based on multiple genotoxicity and carcinogenicity estimates can equal or exceed those obtained by one or two *in vitro* tests, and whether general rules for adaptation of the standard testing regime as described in Annex XI to REACH may be invoked based on such estimates. This must be considered on a case-by-case basis.

(Q)SAR models are continuously updated to improve predictions, with new versions typically released on a yearly basis. It is important to understand the impact of model updates on mutagenicity predictions over time. Such analysis has been done for instance by Hasselgren *et al.* (2020) on computational methods used for the prediction of the mutagenic properties of drug impurities.

Testing data on mutagenicity

Evaluation of genotoxicity test data should be made with care.

Each test guideline contains **criteria for the acceptability of the study** based on important parameters related to the study design and test conditions (e.g. acceptable cell type or animal species, number of cells used and scored or animals tested per group, dose/concentrations levels and the number of test dose/concentrations, recommended negative and positive controls, treatment schedule, exposure and sampling time(s), acceptable levels of (cyto)toxicity, evidence of target tissue exposure, laboratory proficiency demonstration) and **criteria for the evaluation and interpretation of results** (definition of clearly positive and clearly negative responses based on e.g. statistical analysis or threshold values, comparison with historical control ranges for the negative and positive controls).

In addition, further aspects described below need to be considered to determine the validity of study results.

Regarding ***positive* findings**, particular points should be taken into account:

- are the testing conditions (*e.g.* pH, osmolality, precipitates) in *in vitro* mammalian cell assays relevant to the conditions *in vivo*?
- for studies *in vitro*, factors known to influence the specificity of mammalian cell assays such as the cell line used, the top concentration tested, the toxicity measure used or the metabolic activation system used, should be taken into consideration.

- responses generated only at highly toxic/cytotoxic doses or concentrations should be interpreted with caution (*i.e.* taking into account the criteria defined in OECD guidelines).
- the presence or absence of a dose (concentration)-response relationship should be considered.

Particular points to take into account when evaluating ***negative*** **test results** include:

- the doses or concentrations of test substance used (were they high enough? For studies *in vivo*, was a sufficienlty high dose level inducing signs of toxicity used? For studies *in vitro*, was a sufficient level of cytotoxicity reached?).
- was the test system used sensitive to the nature of the genotoxic changes that might have been expected? For example, some *in vitro* test systems will be sensitive to point mutations and small deletions but not to mutagenic events that create large deletions.
- the volatility of the test substance (were concentrations maintained in tests conducted *in vitro*?).
- for studies *in vitro*, the possibility of metabolism not being appropriate in the test system including studies in extra-hepatic organs.
- was the test substance taken up by the test system used for *in vitro* studies?
- was a sufficient number of cells scored/sampled for studies *in vitro*? Has the appropriate number of samples/technical replicates been scored to support statistical significance of the putative negative result?
- for studies *in vivo*, was(were) the most appropriate tissue(s) sampled? Did the substance reach the target organ? Or was the substance only expected to act at the site of contact due to its high reactivity or insufficient systemic availability (taking also toxicokinetic data into consideration, *e.g.* rate of hydrolysis and electrophilicity may be factors that need to be considered)?
- for studies *in vivo*, was sampling appropriate? (Was a sufficient number of animals used? Were sufficient sampling times used? Was a sufficient number of cells scored/sampled?)

Different results between different test systems should be evaluated with respect to their individual significance. Examples of points to be considered are as follows:

- different results obtained in non-mammalian systems and in mammalian cell tests may be addressed by considering possible differences in substance uptake and metabolism, or in genetic material organisation and ability to repair. Although the results of mammalian tests may be considered of higher significance, additional data may be needed to explain differences.
- if the results of indicator tests detecting putative DNA lesions (*e.g.* DNA binding, DNA damage, DNA repair; SCE) are not in agreement with results obtained in tests for mutagenicity, the results of mutagenicity tests are generally of higher significance provided that appropriate mutagenicity tests have been conducted. This is subject to expert judgement.
- if different findings are obtained *in vitro* and *in vivo*, in general, the results of *in vivo* tests indicate a higher degree of reliability. However, for evaluation of *negative* results

in vivo, it should be considered whether the most appropriate tissues were sampled and whether there is adequate evidence of target tissue exposure.

- the sensitivity and specificity of different test systems may vary for different classes of substances. If available testing data for other related substances permit assessment of the performance of different assays for the class of substance under evaluation, the result from the test system known to produce more accurate responses would be given higher priority.

Different results may also be available from the same test, performed by different laboratories or on different occasions. In this case, expert judgement should be used to evaluate the data and reach an overall conclusion. In particular, the quality of each of the studies and of the data provided should be evaluated, with special consideration of the study design, reproducibility of data, dose (concentration)-effect relationships, and biological relevance of the findings. The identity and purity of the test substance may also be a factor to take into account. In the case where an EU/OECD test guideline is available for a test method, the quality of a study using the method is regarded as being higher if it was conducted in compliance with the requirements stated in the test guideline, unless convincing scientific evidence can be provided to justify certain deviations from the standard test guideline for the specific substance evaluated. Furthermore, compared to non GLP-studies, studies compliant with GLP for the same assay generally provide more documentation and details of the study, which are important factors to consider when assessing study reliability/quality.

When assessing the potential mutagenicity of a substance, or considering the need for further testing, data from various tests and genotoxic endpoints may be found. Both the strength and the weight of the evidence should be taken into account. The strongest evidence will be provided by modern, well-conducted studies in line with internationally established test guidelines/methods. For each test type and each genotoxic endpoint, there should be a separate *Weight of Evidence* analysis. It is not unusual for positive evidence of mutagenicity to be found in just one test type or for only one endpoint. In such cases the positive and negative results for different endpoints are not conflicting but illustrate the advantage of using test methods for a variety of genetic alterations to increase the probability of identifying substances with mutagenic potential. Hence, results from methods testing different genotoxic endpoints should not be combined in an overall *Weight of Evidence* analysis but should be subjected to such analysis separately for each endpoint. Based on the whole data set one has to consider whether an appropriate conclusion/assessment can be made or whether there are data gaps. If there are data gaps, further testing should be considered.

R.7.7.4.2 Human data on mutagenicity

Human data have to be assessed carefully on a case-by-case basis. The interpretation of such data requires considerable expertise. Attention should be paid especially to the adequacy of the exposure information, confounding factors, co-exposures and to sources of bias in the study design or incident. The statistical power of the test may also be considered. It may be mentioned that, to date, no germ cell mutagen has been identified based on human data.

R.7.7.4.3 Remaining uncertainty on mutagenicity

Reliable data can be generated from well-designed and conducted studies *in vitro* and *in vivo*. However, due to the lack of human data available and the degree of uncertainty, which is always inherent in testing, a certain level of uncertainty remains when extrapolating these testing data to the effect in humans.

R.7.7.5 Conclusions on mutagenicity

R.7.7.5.1 Concluding on Classification and Labelling

In order to conclude on an appropriate classification and labelling position with regard to mutagenicity, the available data should be considered using the criteria according to Annex I to the CLP Regulation (EC) No 1272/2008 (See also Section 3.5 of the *Guidance on the Application of the CLP criteria*).

R.7.7.5.2 Concluding on suitability for Chemical Safety Assessment

Considerations on dose (concentration)-response shapes and mode of action of mutagenic substances in test systems

Considerations on the dose (concentration)-response relationship and on possible mechanisms of action are important components of a risk assessment. The default assumption for genotoxic substances has for long been that they have a linear dose (concentration)-response relationship. However, this assumption has been challenged by experimental evidence showing that both direct and indirect acting genotoxins can possess non-linear or thresholded dose (concentration)-response curves.

Examples of non-DNA reactive mechanisms that may be demonstrated to lead to genotoxicity *via* non-linear or thresholded dose (concentration)-response relationships include: inhibition of DNA synthesis, alterations in DNA repair, overloading of defence mechanisms (anti-oxidants or metal homeostatic controls), interaction with microtubule assembly leading to aneuploidy, topoisomerase inhibition, high cytotoxicity, metabolic overload and physiological perturbations (*e.g.* induction of erythropoeisis). The mechanisms underlying non-linear or thresholded dose (concentration)-response relationships for some DNA reactive genotoxic substances like alkylating agents seem linked to DNA repair capacity.

Assessment of the significance to be assigned to genotoxic responses mediated by such mechanisms would include an assessment of whether the underlying mechanism can be induced at substance concentrations that can be expected to occur under relevant *in vivo* conditions.

In general, several concentrations/doses are tested in genotoxicity assays. At least three experimental concentrations/doses have to be tested as recommended in the OECD test guidelines for genotoxicity. Determination of experimental dose (concentration)-dependent effects is one of several pieces of experimental information that are important to assess the genotoxic potential of a substance, and may be used as indicated below. It should be recognised that not all of these considerations may be applicable to *in vivo* data.

- the OECD overview document of the genotoxicity test guidelines (OECD, 2017) lists the relevant criteria to be fulfilled for a result to be considered as a clear positive: (i) the increase in genotoxic response is concentration- or dose-related, (ii) at least one of the data points exhibits a statistically significant increase compared to the concurrent negative control, and (iii) the statistically significant result is outside the distribution of the historical negative control data (*e.g.* 95% confidence interval). In practice, the criterion for dose (concentration)-related increase in genotoxicity will be most helpful for *in vitro* tests, but care is needed to check for cytotoxicity or cell cycle delay which may cause deviations from a dose (concentration)-response related effect in some experimental systems.

- genotoxicity tests are not designed to support derivation of no effect levels. However, the lowest dose with an observed effect (*i.e.* the Lowest Observed Effect Dose or LOED) may, on certain occasions, be a helpful tool in risk assessment. This is true specifically for genotoxic effects caused by thresholded mechanisms, like, *e.g.* aneugenicity. Further, it can give an indication of the mutagenic potency of the substance in the test

at issue. Modified studies, with additional dose or concentration points and improved statistical power may be useful in this regard. The Benchmark dose (BMD) approach presents several advantages over the NOED/LOED approach and can be used as an alternative strategy for dose (concentration)-response assessment (see the *Guidance on IR&CSA, Chapter R.8*).

- unusual shapes of dose (concentration)-response curves may contribute to the identification of specific mechanisms of genotoxicity. For example, extremely steep increases suggest an indirect mode of action or a metabolic switching which could be confirmed by further investigation.

Considerations on genetic risks associated with human exposure to mutagenic substances

There are no officially adopted methods for estimating health risks associated with (low) exposures of humans to mutagens. In fact, most – if not all tests used today – are developed and applied to identify mutagenic properties of the substance, *i.e.* identification of the mutagenic hazard *per se*. In today's regulatory practice, the assessment of human health risks from exposure to mutagenic substances that are also carcinogenic is considered to be covered by assessing and regulating the carcinogenic risks of these agents. The reason for this is that mutagenic events underlie these carcinogenic effects. Therefore, mutagenicity data is not used for deriving dose descriptors for risk assessment purposes and the reader is referred to this aspect in Section **Error! Reference source not found.** (Carcinogenicity) for guidance on how to assess the chemical safety for mutagenic carcinogens.

R.7.7.5.3 Information not adequate

A *Weight of Evidence* approach, comparing available adequate information with the tonnage-triggered information requirements by REACH, may result in the conclusion that the requirements are not fulfilled. In order to proceed with gathering further information, the following testing strategy can be adopted:

R.7.7.6 Integrated Testing Strategy (ITS) for mutagenicity

R.7.7.6.1 Objective / General principles

This testing strategy describes a flexible, stepwise approach for hazard identification with regard to the mutagenic potential of substances, so that sufficient data may be obtained for adequate risk characterisation including classification and labelling. It serves to help minimise the use of animals and costs as far as it is consistent with scientific rigour. A flow chart of the testing strategy is presented in **Error! Reference source not found.** and recommendations on follow-up procedures based on different testing data sets are given in Table R.7.7–6. As noted later in this section, deviations from this strategy may be considered if existing data, for instance on analogue substances or on specific mechanisms of action of the substance itself, indicate that alternative testing strategies would yield results with greater sensitivity and specificity for mutagenicity *in vivo*.

The strategy defines a level of information that is considered sufficient to provide adequate reassurance about the potential mutagenicity of most substances. As described below, this level of information will be required for most substances at the Annex VIII tonnage level specified in REACH, although circumstances are described when the data may be required for substances at Annex VII.

For some substances, relevant data from other sources/tests may also be available (*e.g.* physico-chemical, toxicokinetic, and toxicodynamic parameters and other toxicity data; data on well-investigated, structurally similar, substances). These should be reviewed because, sometimes, they may indicate that either more or less genotoxicity studies are needed on the

substance than defined by standard information requirements, *i.e.* they may allow tailored testing/selection of test systems. For example, bacterial mutagenesis assays of inorganic metal compounds are frequently negative due to limited capacity for uptake of metal ions and/or the induction of large DNA deletions by metals in bacteria potentially leading to an increased death rate in mutants. The high prevalence of false negatives for metal compounds might suggest that mutagenesis assays with mammalian cells, as opposed to bacterial cells, would be the preferred starting point for testing for this class of Annex VII substances.

In summary, a key concept of the strategy is that initial genotoxicity tests and testing guidelines/methods should be selected with due consideration to existing data in order to establish the most appropriate testing strategy for the class of compound under evaluation. Even then, initial testing may not always give adequate information and further testing may sometimes be considered necessary in the light of all available relevant information on the substance. Further testing will normally be required for substances which give rise to reliable positive results in any of the *in vitro* tests.

If negative results are available from adequate genotoxicity studies conducted in appropriate test systems, there may be no requirement to conduct additional genotoxicity tests.

Provided that appropriate risk management measures are implemented, substances known to cause germ cell mutagenicity, i.e. meeting the criteria for classification as germ cell mutagen category 1A or 1B, or known to be genotoxic carcinogens, i.e. meeting the criteria for classification both in category 1A, 1B or 2 for germ cell mutagenicity and category 1A or 1B for carcinogenicity according to the CLP Regulation (EC) No 1272/2008[2], will usually not require additional testing in order to meet the requirements of Annexes VII to X for mutagenicity.

Similarly, the carcinogenicity study to meet the requirements of Annex X (see Section R.7.7.2 of this Guidance) and the reproductive toxicity studies to meet the requirements of Annexes VIII to X (see Section R.7.7.6 of this Guidance) may be omitted for substances meeting the CLP criteria for classification in category 1A or 1B for germ cell mutagenicity, provided that appropriate risk management measures are implemented.

Further information on classification according to the CLP Regulation can be found in the *Guidance on the Application of the CLP criteria.*

In cases where a registrant is unsure of the formal position on the classification of a substance or wishes to make a harmonised classification (CLH) proposal themselves, advice could be sought from an appropriate regulatory body. More information on the CLH process, harmonised entries in Annex VI to CLP and adopted RAC opinions on CLH proposals, ongoing CLH consultations, and the registry of CLH intentions can be found on the ECHA website (https://echa.europa.eu/regulations/clp/harmonised-classification-and-labelling).

In case additional testing is needed to meet the requirements of Annex IX or Annex X, or if the additional test is mentioned in Annex IX or Annex X but triggered at Annex VII or Annex VIII, the registrant must first submit a testing proposal to the European Chemicals Agency (ECHA) and obtain prior authorisation before any testing can be initiated.

It should also be noted that recommendations on a strategy for genotoxicity testing have also been published by other authoritative organisations (EFSA, 2011; EMA, 2012). These

[2] At the time of writing of this Guidance, discussion is ongoing on the possible revision of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) criteria for germ cell mutagenicity classification. As it is not known yet whether and/or when some revised criteria will be adopted under GHS or how and when they will be implemented into the CLP Regulation, registrants are advised to regularly check the latest developments on UNECE (https://unece.org/about-ghs) and ECHA (https://echa.europa.eu/regulations/clp/legislation) websites. Only subsequent changes to the CLP Regulation, however, would be legally binding.

strategies are based either on a stepwise approach or on a test-battery approach. Their principle is basically similar to the one detailed in this Guidance, *i.e.* the use of different pieces of information, including non-testing data and results from *in vitro* and *in vivo* testing, for a comprehensive assessment of the genotoxic potential a substance since no single test is capable of detecting all genotoxic mechanisms. However, as these strategies aim at serving different regulations and purposes, some differences can exist between them, in particular regarding the list of *in vitro* and *in vivo* tests recommended and the way they should be used. For instance, while EFSA recommend the use of a core two-test battery for *in vitro* genotoxicity assessment (*i.e.* a bacterial reverse mutation test and an *in vitro* micronucleus test), the REACH Regulation and this Guidance state the *in vitro* mammalian cell gene mutation test as a legal requirement in addition to the Ames test and the *in vitro* cytogenicity test if both tests are negative. Moreover, the *in vitro* chromosome aberration test is considered as a possible alternative to the *in vitro* micronucleus test under REACH while it is now generally agreed that these tests are not equivalent since the *in vitro* chromosome aberration test is not optimal to measure numerical chromosome aberrations. Although this Guidance aims at implementing the latest scientific developments in the field of genotoxicity testing, its main goal is to provide advice and support to the registrant in complying with the legal requirements under REACH and is thus in line with this Regulation.

R.7.7.6.2 Preliminary considerations

For a comprehensive coverage of the potential mutagenicity of a substance, information on the different mutagenicity endpoints, i.e gene mutation (base substitutions and deletions/additions), structural chromosome aberration (breaks and rearrangements) and numerical chromosome aberration (loss or gain of chromosomes, defined as aneuploidy), is required. This may be obtained from available data or new tests on the substance itself or on analogue substances (by chemical grouping or read-across approaches) or, sometimes, by predictions using appropriate *in silico* techniques (e.g. (Q)SAR approaches) in accordance with Annex XI to REACH.

It is important that the available information on the physico-chemical properties of the test substance be taken into account before devising an appropriate testing strategy. Such information may have an impact on the selection of the test systems to be employed and/or (the modifications to) the test protocols to be used. The chemical structure of a substance can provide information for an initial assessment of the mutagenic potential. The need for special testing in relation to photomutagenicity may be indicated in some specific cases by the structure of a molecule, its light absorbing potential, or its potential to be photoactivated. By using expert judgement, it may be possible to identify whether a substance, or a potential metabolite of a substance, shares or does not share structural characteristics with known mutagens. This can be used to justify a higher or lower level of priority for the characterisation of the mutagenic potential of a substance. Where the level of evidence for mutagenicity is particularly strong, it may be possible to make a conclusive hazard assessment on the basis of structure-activity relationships alone in accordance with Annex XI to REACH without additional testing: in this case, the registrant still has to provide sufficient information to meet the requirements of Annexes VII to X but he may, if scientifically justified and duly documented in the registration dossier, invoke the general rules of Annex XI for adaptation of the standard testing regime by demonstrating, *inter alia*, that the results he wishes to use instead of testing in that context are adequate for the purpose of classification and labelling and/or risk assessment.

In vitro tests are particularly useful for gaining an understanding of the potential mutagenicity of a substance and they have a critical role in this testing strategy. They, however, have limitations. Animal tests will, in general, be needed for the clarification of the relevance of positive *in vitro* findings and in case of specific metabolic pathways that cannot be simulated adequately *in vitro*.

The toxicokinetic and toxicodynamic properties of the test substance should be considered before undertaking, or appraising, animal tests. Understanding these properties will enable appropriate protocols for the standard tests to be developed, especially with respect to tissue(s) to be investigated, the route of substance administration and the highest dose tested. If little is understood about the systemic availability of a test substance at this stage, toxicokinetic investigations or modelling may be necessary.

Certain substances may need special consideration, such as highly electrophilic substances that give positive results *in vitro*, particularly in the absence of metabolic activation. Although these substances may react with proteins and water *in vivo* and thus be rendered inactive towards many tissues, they may be able to express their mutagenic potential at the initial site of contact with the body. Consequently, the use of test methods such as the comet assay or the gene mutation assays using transgenic animals that can be applied to the respiratory tract, the upper gastrointestinal tract and skin may be appropriate. It is possible that specialised test methods will need to be applied in these circumstances, and that these may not have recognised, internationally validated, test guidelines. The validity and utility of such tests and the selection of protocols should be assessed by appropriate experts or authorities on a case-by-case basis.

Criteria for the evaluation and interpretation of results (e.g. how to define clear positive and clear negative results) are normally defined in the testing guidelines/methods. There is no requirement for verification of a clear positive or clear negative result. In cases where the response is neither clearly negative nor clearly positive and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgement and/or further investigations. A substance giving such a response should be reinvestigated immediately, normally using the same test method, but varying the conditions to obtain conclusive results. Only if, even after further investigations, the data set precludes coming to a conclusion of a positive or negative result, will the result be concluded as equivocal. Wherever possible, clear results should be obtained for one step in the strategic procedure before going on to the next. In cases where this does not prove to be possible and the study is inconclusive as a consequence of *e.g.* some limitation of the test or procedure, a further test should be conducted in accordance with the strategy.

Tests need not be performed if they can be adapted under Column 2 of Annexes VII-X or under Annex XI to REACH. However, according to REACH article 13(3), tests as described in OECD Guidelines or Regulation (EC) No 440/2008 must be used when data generation is required. Alternatively, for other tests, up-to-date protocols defined by internationally recognised groups of experts, *e.g.* International Workshop on Genotoxicity Testing (IWGT, under the umbrella of the International Association of Environmental Mutagen Societies), may be used under REACH Annex XI provided that the tests are scientifically justified. It is essential that all tests be conducted according to rigorous protocols in order to maximise the potential for detecting a mutagenic response, to ensure that negative results can be accepted with confidence and that results are comparable when tests are conducted in different laboratories.

If a registrant wishes to undertake any tests mentioned in Annex IX or Annex X that require the use of vertebrate animals, then there is a need to make a testing proposal to ECHA first. Testing may only be undertaken after ECHA has accepted the testing proposal in a formal decision.

R.7.7.6.3 Testing strategy for mutagenicity

Standard information requirement at Annex VII

A preliminary assessment of mutagenicity is required for substances at the REACH Annex VII tonnage level (1 to 10 t/y). All available information must be included in the dossier but, as a minimum, there must be data from a gene mutation test in bacteria under Annex VII, section

8.4.1, unless it can be adapted under Column 2 of Annex VII or under Annex XI to REACH. For instance, in case the gene mutation test in bacteria is not applicable to the substance (e.g. for substances with significant toxicity to bacteria, not taken up by bacteria, or for nanoforms) and/or technically not possible, an *in vitro* mammalian cell gene mutation test is required as an alternative test according to Annex VII, sections 8.4 and 8.4.1, column 2.

Under Annex VII, section 8.4, column 2, when the result of the gene mutation test in bacteria, or the gene mutation test in mammalian cells when triggered or available, is positive and raises a concern for gene mutation, an *in vitro* cytogenicity test in mammalian cells as described under Annex VIII, section 8.4.2 must be performed. This is important to also clarify the potential of the substance to also induce structural and numerical chromosome aberrations. According to Annex VIII, section 8.4.2, the following two test methods can be used:

- The *in vitro* chromosome aberration test (OECD TG 473). This is a cytogenetic assay for structural chromosome aberrations using metaphase analysis. An increase in polyploidy may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not routinely used for that purpose. Accordingly, this test guideline is not designed to measure numerical aberrations.

- The *in vitro* micronucleus test (OECD TG 487). This is a cytogenetic assay that has the advantage of detecting not only structural chromosomal aberrations (clastogenicity) but also numerical chromosome aberrations (aneuploidy). Use of a cytokinesis block, fluorescence *in situ* hybridisation with probes for centromeric DNA, or immunochemical labelling of kinetochore proteins can provide information on the mechanisms of chromosome damage and micronucleus formation. The labelling and hybridisation procedures can enable aneugens to be distinguished from clastogens. This may sometimes be useful for risk characterisation. If a substance is demonstrated to be an aneugen, it is assumed that its genotoxicity is thresholded, in contrast to non-thresholded genotoxicity. Both types of genotoxicity mechanisms trigger different ways to perform risk assessment.

As it can detect both structural and numerical chromosomal aberrations, the *in vitro* micronucleus test (OECD TG 487) is considered as the most appropriate *in vitro* cytogenicity test. If the result of the *in vitro* micronucleus test is positive, the aneugenic potential of the substance must be assessed by using one of the centromere labelling or hybridisation procedures described in OECD TG 487 to determine whether the increase in the number of micronuclei is the result of clastogenic events (i.e. micronuclei contain chromosome fragments) and/or aneugenic events (i.e. micronuclei contain whole chromosomes).

Based on the results of the above *in vitro* genotoxicity tests, appropriate *in vivo* follow-up testing in somatic cells as described under Annex IX, section 8.4.4 must be conducted to clarify the concerns identified *in vitro*, *i.e.* the gene mutation concern in case of positive results in the gene mutation test in bacteria or in mammalian cells, the chromosomal aberration concern in case of positive results in the *in vitro* cytogenicity test, or both concerns.

It should also be noted that, in case one or several of the required *in vitro* genotoxicity tests is/are not applicable to the substance and/or technically not possible, *in vivo* testing in somatic cells is required to clarify the concern(s) that cannot be investigated *in vitro.* Substances which, by virtue of, for example, their physico-chemical characteristics, chemical reactivity or toxicity cannot be tested in one or more of the *in vitro* tests should be considered on a case-by-case basis. In the same way, it may not always be possible with the S9 fraction used *in vitro* to mimic the *in vivo* metabolism of some substances, and the relevance of the *in vitro* negative results for those substances should be evaluated case by case. In addition, equivocal *in vitro* results or different results from different *in vitro* studies may require the consideration

of further testing to reach a clear conclusion on mutagenicity. For those types of cases, expert judgement may be needed to determine whether *in vivo* testing is appropriate.

Even if the follow-up *in vivo* somatic cell study is triggered at Annex VII, the registrant must first submit a testing proposal to ECHA and obtain prior authorisation before the *in vivo* study can be initiated.

Standard information requirement at Annex VIII

For a comprehensive coverage of the potential mutagenicity of a substance, information on gene mutations, and structural and numerical chromosome aberrations is required for substances at the Annex VIII tonnage level of REACH.

Under Annex VIII, section 8.4.2, an *in vitro* cytogenicity test, i.e. an *in vitro* chromosome aberration test (OECD TG 473) or an *in vitro* micronucleus test (OECD TG 487) is required. As described above, the *in vitro* micronucleus test is the most appropriate *in vitro* cytogenicity test to detect both structural and numerical chromosome aberrations.

According to Annex VIII, section 8.4, column 2, it is possible to present existing data from an *in vivo* cytogenicity test (*i.e.* a study or studies conducted previously) as an alternative to the first *in vitro* mammalian cell test. For instance, if an adequately performed *in vivo* micronucleus test is already available, it may be used to adapt the information requirement for the *in vitro* cytogenicity study in mammalian cells. There may however be specific cases where the *in vitro* mammalian cell test can still be justified even though *in vivo* cytogenicity data exist. For example, in the *in vivo* micronucleus test, certain substances may not reach the bone marrow due to low bioavailability or specific tissue/organ distribution and would result negative. In addition, even if bioavailability of the parent compound in the bone marrow can be demonstrated, a clastogen requiring liver metabolism and for which the reactive metabolites formed are too short-lived to reach the bone marrow could give a negative result in the *in vivo* micronucleus test. In these cases, *in vitro* testing could provide useful information on the mode of action of the substance, *e.g.* to understand whether the substance is clastogenic (or aneugenic) *in vitro*, and whether it requires a specific metabolism to be genotoxic. Justification of *in vitro* testing when reliable *in vivo* data already exist should be considered on a case-by-case basis.

Under Annex VIII, section 8.4.3, an *in vitro* gene mutation study in mammalian cells (OECD TG 476 or OECD TG 490) is also required when the results of the bacterial gene mutation test and the first study in mammalian cells (*i.e.* an *in vitro* chromosome aberration test or an *in vitro* micronucleus test) are negative. This is to detect *in vitro* mutagens that give negative results in the other two tests. For substances for which an *in vitro* gene mutation study in mammalian cells has already been conducted, this information must always be provided in the dossier as part of the overall *Weight of Evidence* for mutagenicity with reference to induction of gene mutations in mammalian cells, whatever the gene mutation study in bacteria and the *in vitro* cytogenicity test results are.

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According to Annex VIII, section 8.4, column 2, the *in vitro* mammalian cell gene mutation test will usually not be required if adequate information is available from a reliable *in vivo* gene mutation study. Such information may come from a TGR or Pig-a gene mutation assay, if target tissue exposure to the substance or its metabolites is demonstrated. A comet assay may also be adequate even if this test is an indicator assay detecting putative DNA lesions and not gene mutations *per se*, as it can detect substances causing gene mutations *in vivo*. The use of existing UDS studies should be justified on a case-by-case basis. For example, UDS test results should be used only when the liver is a target organ, since the UDS is restricted to the detection of primary DNA repair in liver cells.

Provided all the required *in vitro* tests have given negative results, no *in vivo* test will normally be required to fulfil the standard information requirements at Annex VIII. However, according

to Annex VIII, section 8.4, column 2 and as described above for Annex VII, there may be rare occasions when it is appropriate to conduct testing *in vivo*, for example when one or several of the required *in vitro* genotoxicity tests is/are not applicable to the substance and it is not possible technically to perform satisfactory testing *in vitro*. For those types of cases, expert judgement is needed to determine which *in vivo* testing is appropriate to clarify the concern(s) that cannot be investigated *in vitro*.

Requirement for testing beyond the standard levels specified for Annexes VII and VIII

Introductory comments

According to Annexes VII and VIII, section 8.4, column 2, concerns raised by positive results from *in vitro* tests usually require further testing. The chemistry of the substance, data on analogous substances, toxicokinetic and toxicodynamic data, and other toxicity data will also influence the timing and pattern of further testing. Unless there are appropriate results from an *in vivo* study already, testing beyond the standard set of *in vitro* tests is normally first directed towards investigating the potential for mutagenicity in somatic cells *in vivo* as described under Annex IX, section 8.4.4.

Positive genotoxicity results in somatic cells *in vivo* normally constitute the trigger for investigation of potential expression of genotoxicity in germ cells at Annexes IX and X, but not at Annexes VII and VIII.

However, the TGR assays give the possibility to include sampling of somatic and male germ cells in a single study providing appropriate sampling times are used (see OECD TG 488 for details). Therefore, to avoid unnecessary testing of vertebrate animals and additional costs, it is recommended to include the collection of germ cell samples in any testing proposal for the TGR assays, even at Annexes VII and VIII, and even in case the test is proposed primarily to investigate somatic tissues. Germ cell samples should be appropriately stored for later analysis.

The comet assay can also be conducted on both somatic and germ cells although, as described in OECD TG 489, it is currently not recommended for mature germ cell testing. However, in case the comet assay is proposed for somatic cell investigation, male gonadal cells can be collected in the same study and slides prepared for later analysis. Since gonads contain a mixture of somatic and germ cells, positive results in male gonadal cells are not necessarily reflective of germ cell damage but they indicate that the substance and/or its metabolites have reached the gonad and induced a genotoxic effect in this compartment.

In case of positive results in any of the somatic tissues tested in the TGR or the comet assay, analysis of germ cell samples may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation.

Substances that are negative in the standard set of in vitro tests

In general, substances that are negative in the full set of *in vitro* tests specified in REACH Annexes VII and VIII are considered to be non-genotoxic. There are only a very limited number of substances that have been found to be genotoxic *in vivo*, but not in the standard *in vitro* tests. Most of these are pharmaceuticals designed to affect pathways of cellular regulation, including cell cycle regulation, and this evidence is judged insufficient to justify routine *in vivo* testing of industrial chemicals. However, occasionally, knowledge about the metabolic profile of a substance may indicate that the standard *in vitro* tests are not able to detect a potential genotoxic effect and a further *in vitro* test, or an *in vivo* test, may be needed in order to ensure mutagenicity potential is adequately explored (*e.g.* use of an alternative to rat liver S9 mix, a reducing system, a metabolically active cell line, or genetically engineered cell lines might be judged appropriate).

Substances for which an in vitro test is positive

REACH Annex VII substances for which only a bacterial gene mutation test has been conducted and for which the result is positive should be studied further *in vitro* and *in vivo*, according to the requirements of Annex VII, column 2. Further *in vitro* cytogenicity testing will first be needed to determine whether there is also a chromosomal aberration concern in addition to the gene mutation concern raised by the positive gene mutation test in bacteria. Further *in vivo* testing will then be required to address the concern(s) identified *in vitro*. Available *in vitro* gene mutation study results in mammalian cells must always be provided in the dossier and can also trigger further *in vitro* and *in vivo* testing in case they are positive, even if the gene mutation study in bacteria is negative.

At REACH Annex VIII, following a positive result giving rise to concern in any of the *in vitro* mutagenicity tests in bacteria or mammalian cells referred to in Annexes VII and VIII, appropriate *in vivo* testing in somatic cells is required to ascertain whether this genotoxic potential can be expressed *in vivo*. The *in vivo* study must address the concern(s) identified *in vitro*, i.e. the gene mutation concern, the chromosomal aberration concern, or both concerns..

It should be noted that, where further testing at Annexes VII and VIII involves tests mentioned in Annexes IX or X, such as *in vivo* somatic cell genotoxicity studies, testing proposals must be submitted by the registrant and accepted by ECHA in a formal decision before testing can be initiated.

Standard information requirement according to Annexes IX and X

According to the requirements of Annexes IX and X, section 8.4.4, if there is a positive result giving rise to concern in any of the *in vitro* studies from Annex VII or VIII and there are no appropriate results available from an *in vivo* study already, an appropriate *in vivo* somatic cell genotoxicity study should be proposed. The *in vivo* study must address the concern(s) identified *in vitro*, i.e. the gene mutation concern, the chromosomal aberration concern, or both concerns.

In vivo somatic cell genotoxicity test selection

Before any decisions are made about the need for *in vivo* testing, a review of the *in vitro* test results and all available information on the toxicokinetic and toxicodynamic profile of the test substance is needed. A particular *in vivo* test should be conducted only when it can be reasonably expected from all the properties of the test substance and the proposed test protocol that the specific target tissue will be adequately exposed to the test substance and/or its metabolites.

Provided that evidence for adequate availability of the substance or its metabolites to the target tissue/cells specified in the corresponding test guidelines is shown, there are several options for the *in vivo* testing:

- A mammalian erythrocyte (bone marrow or peripheral blood) micronucleus test (OECD TG 474). Compared to the mammalian bone marrow chromosomal aberration test, the micronucleus test has the advantage of detecting not only structural chromosomal aberrations (clastogenicity) but also numerical chromosomal aberrations (aneuploidy) provided that appropriate DNA staining has been applied. Bone marrow is the target tissue and evidence of bone marrow exposure to the substance or its metabolites must be demonstrated.

- A mammalian bone marrow chromosomal aberration test (OECD TG 475). This test is optimised to detect structural chromosomal aberrations but not numerical chromosomal aberrations. Evidence of bone marrow exposure to the substance or its metabolites is required.

- A transgenic rodent (TGR) somatic and germ cell gene mutation assays (OECD TG 488). TGR assays measure gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi- assay models) using reporter genes present in every tissue. In contrast to the in vivo micronucleus test, and in vivo chromosomal aberration test and Pig-a assay, this assay has the advantage of not being restricted to bone marrow cells. In principle every tissue can be sampled, including target tissues and specific site of contact tissues, provided adequate exposure to the substance or its metabolites occurred.

- A mammalian erythrocyte Pig-a gene mutation assay (OECD TG 470). The Pig-a assay measures gene mutations induced in bone marrow erythroid precursor cells and requires only small volumes of peripheral blood without the need to euthanise the animals. The test can identify substances that cause gene mutations in these precursor cells, which are reflected in erythrocytes sampled from peripheral blood cells of animals, usually rodents. Evidence of bone marrow exposure to the substance or its metabolites must be demonstrated.

- A comet (single cell gel electrophoresis) assay (OECD TG 489). The comet assay is an indicator test which detects DNA strand breaks and alkali labile DNA lesions. Although this test is not a mutagenicity test, it is able to detect substances that induce chromosomal aberrations and/or gene mutations. In contrast to the above-mentioned *in vivo* micronucleus test, *in vivo* chromosome aberration test and Pig-a assay, this assay has the advantage of not being restricted to bone marrow cells. In principle every tissue from which single cell or nuclei suspensions can be prepared can be sampled, including specific site of contact tissues, provided adequate exposure to the substance or its metabolites occured.

- Availalable data from other DNA strand breakage assays may be provided in the dossier. All DNA strand break assays should be considered as indicator tests, as they do not detect permanent changes to DNA.

- A rat liver Unscheduled DNA synthesis (UDS) test (OECD TG 486). The UDS test is an indicator test measuring DNA repair of primary damage in liver cells but not a surrogate test for gene mutations *per se*. The UDS test can detect some substances that induce *in vivo* gene mutation because this assay is sensitive to some (but not all) DNA repair mechanisms. However not all gene mutagens are positive in the UDS test and it is thus useful only for some classes of substances. A positive result in the UDS assay can indicate exposure of the liver DNA and induction of DNA damage by the substance under investigation but it is not sufficient information to conclude on the induction of gene mutation by the substance. A negative result in a UDS assay alone is not a proof that a substance does not induce gene mutation. This test is no longer considered appropriate to generate new information under REACH[3] and the above limitations should be carefully considered when using existing UDS data.

Only the first four options for testing mentioned above can be used directly for providing evidence of *in vivo* mutagenicity in somatic cells: OECD TGs 474 and 475 for chromosomal aberration, and OECD TGs 488 and 470 for gene mutation. The other test methods are genotoxicity tests and their results should be assessed together with specific supporting information, for example results from *in vitro* mutagenicity studies, to allow making definitive conclusions about *in vivo* mutagenicity and lack thereof.

[3] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

Any one of the above tests, except the UDS test, may be conducted, but this has to be decided using expert judgement on a case-by-case basis. The nature of the original *in vitro* response(s) (*i.e.* gene mutation, structural or numerical chromosome aberration) must be considered when selecting the follow-up *in vivo* study. Investigation of different endpoints and sampling of different tissues in the same study is also encouraged whenever possible as this would provide a more comprehensive overview of the genotoxic potential of a substance and limit the number of animals used. When combining test methods, care should be taken not to impair the validity of the results from each individual test. Further recommendations and references for combining or integrating different test methods can be found in the respective OECD TGs and the OECD overview document of the genotoxicity test guidelines (OECD, 2017).

For substances showing evidence of *in vitro* clastogenicity, both the *in vivo* micronucleus test and *in vivo* chromosomal aberration test are considered appropriate follow-up tests, provided that bone marrow exposure to the substance or its metabolites occurred. An *in vivo* comet assay may also be appropriate even if this test is an indicator assay detecting putative DNA lesions and not chromosome aberrations *per se*, as it can detect substances causing structural chromosome aberrations *in vivo.* However, only the *in vivo* micronucleus test is able to detect both clastogens and aneugens. Therefore, if a positive result for chromosome aberrations was obtained in *in vitro* but aneugenicity was not investigated, the rodent micronucleus test would be appropriate to best address clastogenic and aneugenic potentials *in vivo*. In case of positive results in the *in vivo* micronucleus test and if the clastogen/aneugen mode of action has not been investigated in the *in vitro* micronucleus test, one of the centromere labelling or hybridisation procedures described in OECD TG 474 must be used to determine whether the increase in the number of micronuclei is the result of clastogenic events (resulting in chromosome fragment(s) contained in micronuclei) and/or aneugenic events (i.e. micronuclei contain whole chromosome(s)). Moreover, as one limitation of the *in vivo* micronucleus test as described in the OECD TG 474 is that it only investigates the bone marrow, combination with the *in vivo* comet assay is appropriate to detect effects in both distant organs, such as the bone marrow or the liver, and at site(s) of contact, such as the glandular stomach and the duodenum (for the oral route of administration), or the lung (after administration through inhalation). Investigating several genotoxic endpoints and different tissues in a combined study is necessary to reduce the uncertainties of not testing all organs and to generate complementary information that provides a comprehensive overview of the genotoxic potential of a substance.

For substances inducing aneugenic effects but no clastogenic effects *in vitro*, as demonstrated in an *in vitro* micronucleus test, the *in vivo* micronucleus test is the only appropriate follow-up test.

For substances that appear preferentially to induce gene mutations, the TGR assays are the most appropriate and usually preferred tests to follow-up an *in vitro* gene mutation positive result and detect substances that induce gene mutations *in vivo*. With respect to the 3Rs principle and taking into account that a positive result in somatic cells triggers the need to consider the potential for germ cell testing, germ cells must always be collected, if possible, when a TGR study is performed.

The Pig-a assay is another appropriate option to follow-up on *in vitro* gene mutation positive results, provided that bone marrow exposure to the substance or its metabolites occur. One advantage of the assay is the use of blood samples, which facilitates combination with other genotoxicity test methods and integration into repeated dose toxicity studies. However, the applicability of the OECD TG 470 is currently limited to rodent bone marrow erythroid cells. Therefore, bone marrow exposure to the substance or its metabolites is required in the Pig-a assay and it cannot be used to measure mutations in other organs such as the liver, the sites of contact tissues or the germ cells.

The *in vivo* comet assay can also detect substances inducing gene mutations, even if it is not a gene mutation assay but an indicator assay measuring DNA damage. This test can be used to

analyse both site(s) of contact and distant organs, although is the protocol described in the current OECD TG 489 not applicable to mature germ cells.

The rat liver UDS test has a long history of use but is no longer considered appropriate to generate new information under REACH[4]. The sensitivity of the UDS test has been questioned (Kirkland and Speit, 2008) and its lower predictive value towards rodent carcinogens and/or *in vivo* genotoxicants has been confirmed in comparison with the TGR assay (EFSA, 2017). The use of existing UDS data should be justified on a case-by-case basis, and take account of substance-specific considerations. Positive UDS test results demonstrate that the substance (or its metabolites) reached liver cells and induced DNA damage and repair. However, a negative result in the UDS test is not considered sufficient to demonstrate that a substance does not induce gene mutations.

The choice of any of the above assays can be justified only if it can be demonstrated that the tissue(s) studied in the assay is (are) sufficiently exposed to the test substance (or its metabolites). This information can be derived from toxicokinetic data or, in case no toxicokinetic data are available, from the observation of treatment-related effects in the organ of interest. Another type of data that can support evidence of organ exposure is knowledge on the target organ(s) of specific classes of substances (*e.g.* the liver for aromatic amines). The TGR and comet assays offer greater flexibility than the Pig-a assay and the UDS test, most notably with regard to the possibility of selecting a range of tissues for study on the basis of what is known of the toxicokinetics and toxicodynamics of the substance. It should be realised that the UDS and comet tests are indicator assays: the comet assay detects DNA lesions whereas the UDS assay detects DNA repair patches (which depend on the DNA repair pathway involved and the proficiency of the cell type investigated), indirectly showing DNA lesions. In contrast, the TGR and Pig-a assays measure gene mutations, *i.e.* permanent transmissible changes in the DNA. Therefore, in case a positive result is obtained in the comet assay (or UDS test) and the presence or absence of gene mutation effects needs to be confirmed, for instance to support proper classification, a follow-up TGR or Pig-a assay may be required.

For substances inducing both chromosome aberrations and gene mutations *in vitro*, the combination of the *in vivo* micronucleus test and the *in vivo* comet assay in a single study is the most appropriate follow-up option (unless the induced chromosome aberrations are only numerical, in which case the *in vivo* micronucleus test alone is appropriate). The combined study, together with the results of the *in vitro* mutagenicity studies, can be used to make definitive conclusions about the *in vivo* mutagenicity potential of the substance in somatic cells and the underlying mechanisms. Furthermore, the combined study can help limit the number of tests performed and the number of animals used while investigating several (site of contact and distant) tissues and addressing (structural and numerical) chromosomal aberrations as well as gene mutations.

It should be noted that in case of both gene mutation and chromosomal aberration concern, when reliable *in vivo* data exist and already address one of the concerns identified *in vitro*, another study is necessary to investigate the remaining endpoint of concern for an full coverage of the *in vivo* mutagenicity potential of the substance.

For substances inducing gene mutation or chromosomal aberration *in vitro*, and for which no indication of sufficient systemic availability is available, or that are short-lived or reactive, an alternative strategy involving studies to focus on tissues at initial sites of contact with the body, such as the glandular stomach and the duodenum (for the oral route of administration) or the lung (after administration through inhalation), must be considered.

[4] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

Expert judgement should be used on a case-by-case basis to decide which tests are the most appropriate. The main options are the *in vivo* comet assay and the TGR gene mutation assays or a combination of both tests. For any given substance, expert judgement, based on all the available toxicological information, will indicate which of these tests are the most appropriate. The route of exposure should be selected to allow the best possible assessment of the hazard posed to humans. For insoluble substances, the possibility of release of active molecules in the gastrointestinal tract may indicate that a test involving the oral route of administration is particularly appropriate.

Non-standard studies supported by published literature may sometimes be more appropriate and informative than established assays. Guidance from an appropriate expert or authority should be sought before undertaking novel studies. Furthermore, additional data that support or clarify the mechanism of action may justify a decision not to test further.

Additionally, evidence for *in vivo* DNA adduct formation in somatic cells together with positive results from *in vitro* mutagenicity tests are sufficient to conclude that a substance is an *in vivo* somatic cell mutagen. In such cases, positive results from *in vitro* mutagenicity tests may not trigger further *in vivo* somatic tissue testing, and the substance would be classified at least as a category 2 mutagen. The possibility for effects in germ cells would need further investigation (see Section R.7.7.6.3, *Substances that give positive results in an in vivo test for genotoxic effects in somatic cells*).

Test combination and integration and limitation of test animal use

In the framework of the 3Rs principles, the combination of *in vivo* genotoxicity studies or integration of *in vivo* genotoxicity studies into repeated dose toxicity studies, whenever possible and when scientifically justified, is strongly encouraged. All the above-mentioned *in vivo* tests in somatic cells are in principle amenable to such integration, although sufficient experience is not yet available for all the tests. It is possible for two or more endpoints to be combined into a single *in vivo* study, and thereby save on resources and numbers of animals used. As described in OECD TGs 489 and 474, the comet assay and the *in vivo* micronucleus test can be combined into a single acute study, although some modification of treatment and sampling times is needed (Hamada *et al*., 2001; Madrigal-Bujaidar *et al*., 2008; Pfuhler *et al.*, 2009; Bowen *et al*., 2011). These same endpoints can be integrated into repeated dose (*e.g.* 28-day) toxicity studies (Pfuhler *et al*., 2009; Rothfuss *et al.*, 2011; EFSA, 2011). The Pig-a assay can also be integrated into repeated-dose toxicity studies and different protocols exist for combining it with the *in vivo* micronucleus test and comet assay (see paragraphs 7-8 and Annex 2 of OECD TG 470).

To ensure that the number of animals used in somatic cell genotoxicity tests is kept to a minimum, both males and females should not automatically be used. In accordance with standard guidelines, testing in one sex only is possible when the available data on the substance, including for instance data from a range-finding study, do not demonstrate relevant sex-specific differences, such as differences in systemic toxicity, target organ toxicity, metabolism or bioavailability.

As indicated in the OECD overview document of the genotoxicity test guidelines (OECD, 2017) and most of the in vivo test guidelines for genotoxicity testing themselves, concurrent positive and negative control animals should normally be used in every test to confirm the reliability of the method and validity of the results. However, if the test laboratory has demonstrated proficiency in the conduct of the test and has established a historical control database for the tissue(s) of interest, it should be considered:

- whether to use concurrent positive control animals. As described in the guidelines of most of the above *in vivo* tests, the use of a concurrent positive control group may be replaced by appropriately stored samples from previous positive control animals, from the same species and strain, and with similar age as those treated with the test substance (i.e. frozen tissues or DNA samples for the TGR assays, fixed and unstained

slides or cell suspension samples used as scoring controls for the *in vivo* micronucleus test, fixed and unstained slides for the chromosomal aberration test, or blood samples used as flow cytometry standards for the Pig-a assay. When concurrent positive control animals are not included in each study, laboratories should still occasionally perform additional tests with mutagen-treated animals to assure continued proficiency in detecting increases in mutant frequency. It should be noted that, according to OECD TG 489 and the overview document of the genotoxicity test guidelines (OECD, 2017), concurrent positive controls are always necessary when conducting the *in vivo* comet assay, since there is insufficient experience with the longevity of alkali labile DNA sites in storage, no agreed tissue freezing and thawing methodology, and no standard method to assess whether a potentially altered response due to storage may affect the sensitivity of the test.

- whether a concurrent positive control group and a concurrent negative control group are to be used for all time points when multiple sampling times are used (*e.g.* for both the early and late time points in the *in vivo* micronucleus assay, or when single treatment with multiple sampling is used in the *in vivo* comet assay).

Substances that give negative results in an in vivo test for genotoxic effects in somatic cells

If the testing strategy described above has been followed and the first *in vivo* test is negative, the need for a further *in vivo* somatic cell test should be considered. A second *in vivo* test should only then be proposed if it is required to make a conclusion on the genotoxic potential of the substance under investigation, *i.e.,* if the *in vitro* data show the substance to have potential to induce both gene mutations and chromosome aberrations and the first *in vivo* test has not addressed both concerns comprehensively. In this regard, on a case-by-case basis, attention should be paid to the quality and relevance of all the available toxicological data, including the adequacy of target tissue exposure.

For a substance giving negative results in adequately conducted, appropriate *in vivo* test(s), as defined by this strategy, it will normally be possible to conclude that the substance is not an *in vivo* mutagen.

Substances that give positive results in an in vivo test for genotoxic effects in somatic cells

Substances that have given positive results in cytogenetic tests both *in vitro* and *in vivo* must be studied further to establish whether they specifically act as aneugens, and therefore whether thresholds for their genotoxic activity can be identified, if this has not been established adequately already. This should be done using *in vitro* methods and will support risk evaluation. Confirmation of the type of chromosomal aberration induced is also important to decide on appropriate follow-up testing.

According to Annex IX, section 8.4.5 and Annex X, section 8.4.7, *in vivo* germ cell genotoxicity testing is required for substances giving positive results in the *in vivo* genotoxicity test(s) in somatic cells.

Substances registered at Annex VII or VIII which are positive in the *in vivo* genotoxicity test(s) in somatic cells but for which no data on germ cells is available are classified as category 2 mutagens under the CLP Regulation (EC) No 1272/2008 (for detailed information on the criteria for classification of substances for germ cell mutagenicity under the CLP Regulation (EC) No 1272/2008, see Section 3.5 of the *Guidance on the Application of the CLP criteria*).

According to Annexes IX and X, section 8.4, column 2, no further information on germ cell mutagenicity is required for substances known to cause germ cell mutagenicity (i.e. meeting the criteria for classification as germ cell mutagen category 1A or 1B according to the CLP

Regulation[5]) or known to be genotoxic carcinogens (i.e. meeting the CLP criteria for classification both in category 1A, 1B or 2 for germ cell mutagenicity and category 1A or 1B for carcinogenicity). The first step is therefore to make an appraisal of all the available data to determine whether there is sufficient information to conclude that the substance poses a hazard as germ cell mutagen or genotoxic carcinogen. If this is the case, the substance must be classified in the appropriate hazard category(ies), appropriate risk management measures must be implemented and no further testing is justified.

Although the hazard class for mutagenicity primarily refers to germ cells, data showing the induction of genotoxic effects at site of contact tissues by substances for which no indication of sufficient systemic availability or presence in germ cells has been presented are also relevant and considered for classification. For such substances, at least one positive *in vivo* genotoxicity test in somatic cells like an *in vivo* comet assay can lead to classification in Category 2 germ cell mutagens and to the labelling as 'suspected of causing genetic defects' if the positive effect *in vivo* is supported by positive results of *in vitro* mutagenicity tests. Classification as Category 2 germ cell mutagen may also have implications for a potential carcinogenicity classification.

If the appraisal of the mutagenic potential of the substance in germ cells raises a concern, additional investigation are required. However, according to Annexes IX and X, sections 8.4.5 and 8.4.7, column 2, no germ cell study should be conducted if there is clear evidence that neither the substance nor its metabolites will reach the germ cells. Expert judgement is needed at this stage to evaluate the available data on the toxicokinetic and toxicodynamic properties of the test substance. In the event that additional information about the toxicokinetics of the substance might clarify the issue, toxicokinetic investigation (*i.e.* not a full toxicokinetic study) tailored to address this question could be performed.

If specific germ cell testing is to be undertaken, expert judgement should be used to select the most appropriate test strategy. The *in vivo* germ cell study(ies) must address the concern(s) identified in somatic cells, i.e. the gene mutation concern, the chromosomal aberration concern, or both concerns.

Internationally recognised guidelines are available for investigating chromosomal aberrations in rodent spermatogonial cells (OECD TG 483) and for the rodent dominant lethal test (OECD TG 478). Dominant lethal mutations are believed to be primarily due to structural or numerical chromosome aberrations. However, the rodent dominant lethal test is no longer considered appropriate to generate new information under REACH[6].

The TGR assays (OECD TG 488) are the only standard test methods detecting gene mutations in germ cells (with appropriate sampling times as indicated in the OECD TG 488).

Alternatively, other methods can be used if deemed appropriate by expert judgement. .

The *in vivo* comet assay (OECD TG 489) is currently not recommended for germ cell testing, but positive results in male gonadal cells indicate that the substance and/or its metabolites have reached the gonad and can cause mutations to germ cells. This type of supporting evidence, in combination with positive results from an *in vivo* somatic cell mutagenicity test,

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[5] At the time of writing of this Guidance, discussion is ongoing on the possible revision of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) criteria for germ cell mutagenicity classification. As it is not known yet when these revised criteria will be adopted under GHS or how and when they will be implemented into the CLP Regulation, registrants are advised to regularly check the latest developments on UNECE (https://unece.org/about-ghs) and ECHA (https://echa.europa.eu/regulations/clp/legislation) websites.

[6] see also Commission Regulation amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods (europa.eu)

can be sufficient to warrant classification of the substance in category 1B for germ cell mutagenicity.

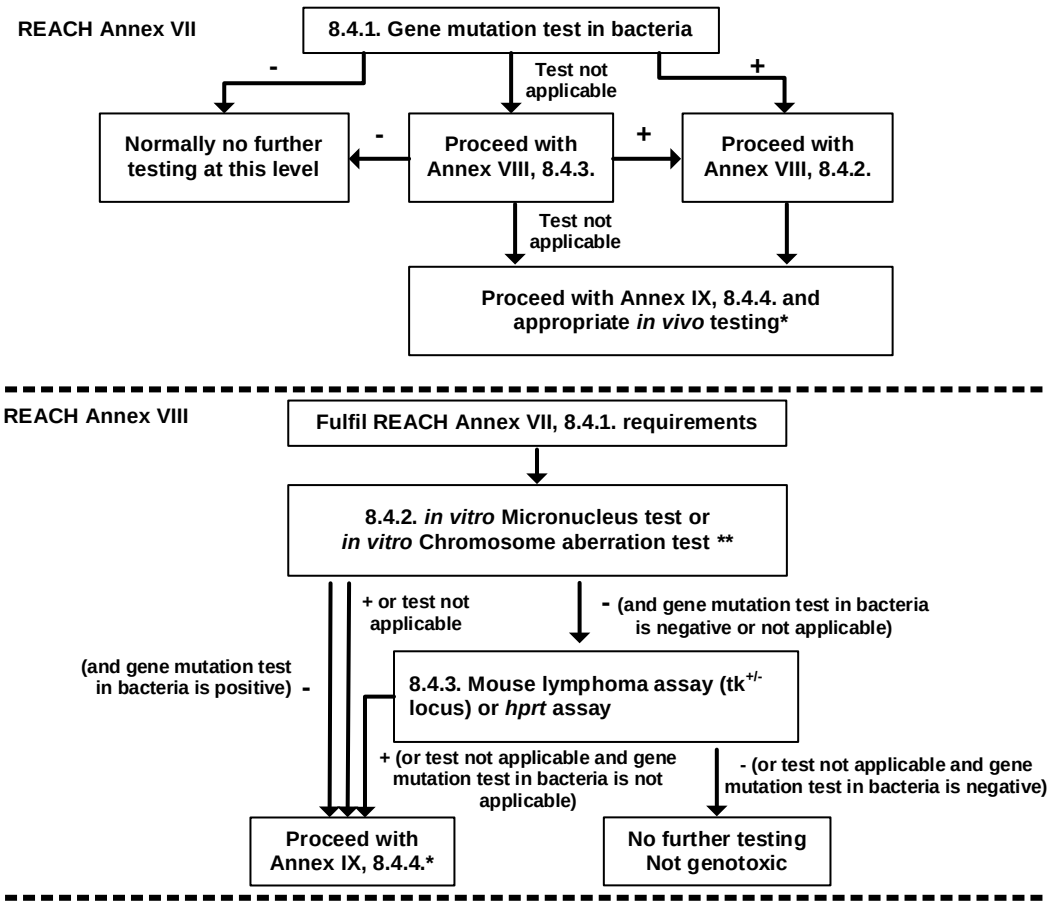
To date, there is no single standard test method or agreed combined study capable of detecting both chromosomal aberrations and gene mutations in germ cells in the same animals. Therefore, when both concerns are raised by the *in vivo* somatic cell test results, it has to be decided case by case which test method(s) to use.

In principle, it is the potential for effects that can be transmitted to the progeny that should be investigated, but tests used historically to investigate transmitted effects (i.e. the heritable translocation test and the specific locus test) use a very large number of animals. They are rarely used nowadays and should normally not be proposed for substances registered under REACH.

In order to minimise animal use, it is recommended to include samples from both relevant somatic tissues and germ cell tissues (*e.g*. testes) in *in vivo* mutagenicity studies: the somatic cell samples can be investigated first and, if they are positive, germ cell tissues can then also be analysed. Finally, the possibility to combine reproductive toxicity testing with *in vivo* mutagenicity testing could be considered.

Figure R.7.7–1 Flow chart of the mutagenicity testing strategy

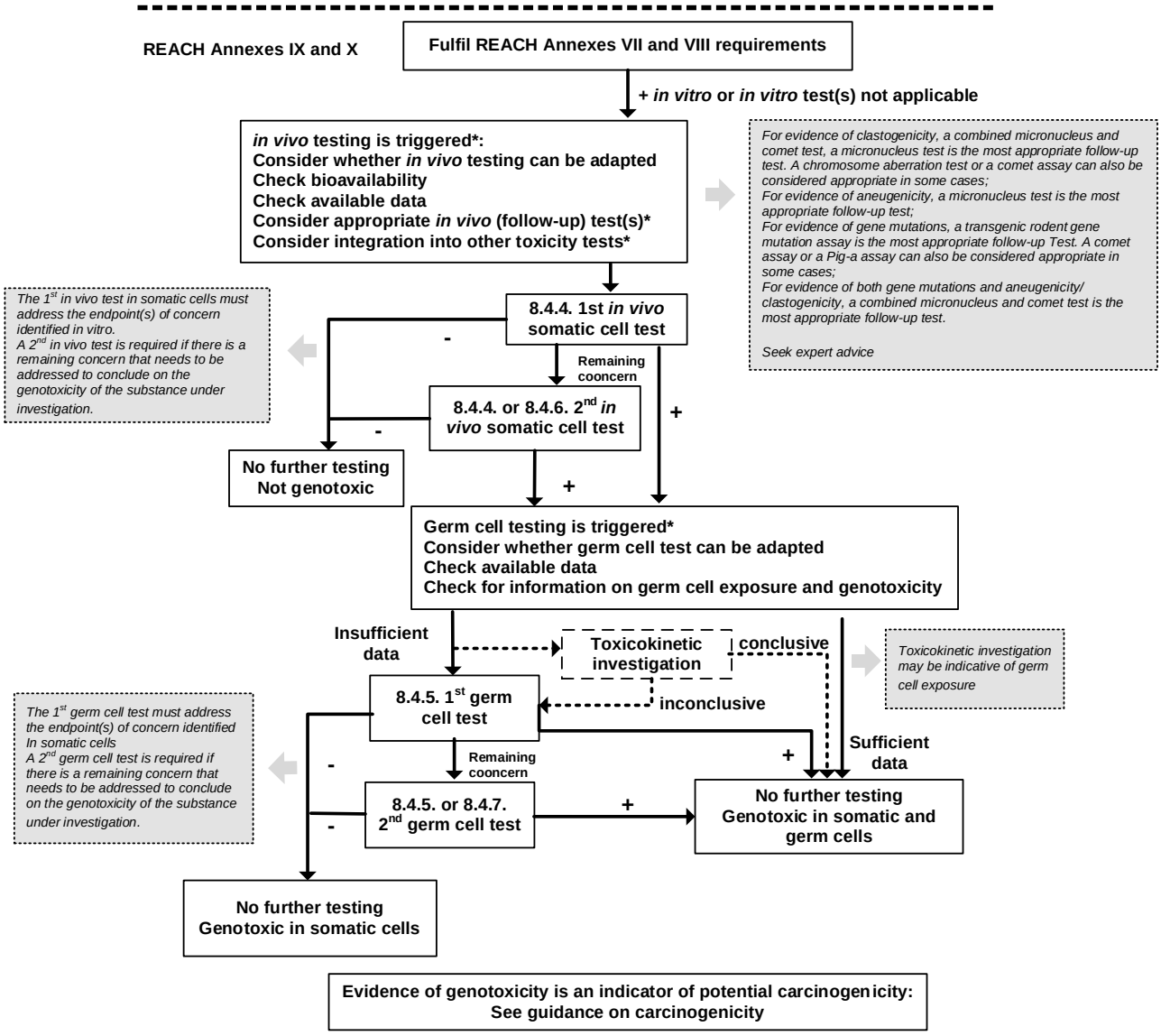
Please note that the present testing strategy is based on a general scenario where no data is available on the substance and new testing has to be conducted. In case relevant and reliable data are already available and can be used to fulfil or adapt some of the information requirements, the strategy should be adjusted accordingly. For further details, please see text in the previous sections of this Guidance.



see Annexes IX and X on next page

* Registrants should note that a testing proposal must be submitted for a test mentioned in Annex IX or X, independently from the registered tonnage. Following examination of such testing proposal ECHA has to approve the test in its evaluation decision before it can be undertaken.

** If a new study needs to be performed to fulfil Annex VIII, section 8.4.2 information requirement, the *in vitro* Micronucleus test is recommended as it can detect both structural and numerical chromosomal aberrations.



* Registrants should note that a testing proposal must be submitted for a test mentioned in Annex IX or X, independently from the registered tonnage. Following examination of such testing proposal ECHA has to approve the test in its evaluation decision before it can be undertaken.

Table R.7.7–6 Examples of different testing data sets and follow-up procedures to conclude on genotoxicity/mutagenicity according to the mutagenicity testing strategy.

Depending on the *in vitro* and *in vivo* test results available and the REACH Annex(es) of interest, further testing may be required to meet the standard information requirements for mutagenicity and allow for a conclusion on genotoxicity/mutagenicity to be reached. Recommendations on what should be

done or particularly looked at in those different cases are mentioned in the table, together with specific rules for adaptation when applicable (for detailed guidance see also main text).

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
1		neg					Annex VII: no further tests are required. Annexes VIII, IX & X: conduct a CAvitro or preferably a MNvitro, and if this test is negative, a GMvitro.	Annex VII: not genotoxic		Annexes VIII, IX & X: Select further tests in such a way that all the tests, together with other available information, enable thorough assessment for gene mutations and for structural and numerical chromosomal aberrations.
2		pos					Annexes VII, VIII, IX & X: Complete *in vitro* testing with a CAvitro or preferably a MNvitro.			Consider need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
3	a	n/a					Annexes VII, VIII, IX & X: conduct a GMvitro.			
	b	n/a	neg							
4		neg	neg				Annex VII: no further tests are required. Annexes VIII, IX & X: conduct a GMvitro.	Annex VII: not genotoxic		Annexes VIII, IX & X: Select tests in such a way that all the tests, together with other available information, enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations.
5	a	neg		neg			Annex VII: no further tests are required. Annexes VIII, IX & X: conduct a CAvitro or preferably a MNvitro	Annex VII: not genotoxic		Annexes VIII, IX & X: Select tests in such a way that all the tests, together with other available information, enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations.
	b	n/a		neg						

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
6	a	neg		pos			Annexes VII, VIII, IX & X: Complete *in vitro* testing with CAvitro or preferably a MNvitro. Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test(s), depending on the result of CAvitro or MNvitro, to investigate: - gene mutations *in vivo* if CAvitro or MNvitro is negative (TGR, comet or Pig-a). - both (structural or numerical) chromosome aberrations and gene mutations if CAvitro or MNvitro is positive (combined MNvivo and comet preferably) If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet (combined or not with MNvivo) is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.		If both genotoxic endpoints are investigated and a first *in vivo* test is available but does not address the genotoxic endpoints comprehensively, a second *in vivo* test to address the remaining genotoxic endpoint must be conducted.	Annexes VIII, IX & X: Select tests in such a way that all the tests, together with other available information, enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations.
	b	n/a		pos						

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
7	a	neg	neg	neg			Annexes VII, VIII, IX & X: no further tests are required.	Not genotoxic		The available case-specific (e.g. metabolic) evidence may, on rare occasions, indicate that *in vitro* testing is inadequate*; in vivo* testing is then needed. Seek expert advice.
	b	n/a	neg	neg						
8		pos	neg				Annexes VII, VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test to investigate gene mutations *in vivo* (TGR, comet or Pig-a). If the TGR is to be conducted on somatic tissues, germ cell samples must be collected, if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.		.	Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider on a case-by-case basis the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
9		neg	pos				Annexes VII, VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select an adequate *in vivo* somatic cell test to investigate structural or numerical chromosome aberrations (combined MNvivo and comet preferably, or CAvivo or comet for *in vitro* clastogens, MNvivo for *in vitro* aneugens) If necessary, seek expert advice.			Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment and to determine whether C&L is justified.
10	a	pos	pos						Both genotoxic endpoints must be investigated. If a first *in vivo* test is available but does not address the genotoxic endpoints comprehensively, a second *in vivo* test to address the remaining genotoxic endpoint must be conducted.	Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
	b	pos	n/a				Annexes VII, VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test(s) to investigate both structural or numerical chromosome aberrations and gene mutations (combined MNvivo and comet preferably) If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet (combined or not with MNvivo) is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.			
	c	n/a	pos	pos						
	d	n/a	n/a	pos						

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
11		n/a	pos				Annexes VII, VIII, IX & X: Conduct a GMvitro. Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test(s) to investigate: - structural or numerical chromosome aberrations if GMvitro is negative (combined MNvivo and comet preferably, or CAvivo or comet for *in vitro* clastogens, MNvivo for *in vitro* aneugens). - both structural or numerical chromosome aberrations and gene mutations if GMvitro is positive (combined MNvivo and comet preferably) If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet (combined or not with MNvivo) is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.		If both genotoxic endpoints are investigated and a first *in vivo* test is available but does not address the genotoxic endpoints comprehensively, a second *in vivo* test to address the remaining genotoxic endpoint must be conducted.	Annexes VIII, IX & X: Select tests in such a way that all the tests, together with other available information, enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations.

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
12	a	neg	neg	pos			Annexes VII, VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select an adequate i*n vivo* somatic cell test to investigate gene mutations *in vivo* (TGR preferably, or comet, or Pig-a). If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.			Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider on a case-by-case basis the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
	b	n/a	neg	pos						

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
13		n/a		n/a			Annexes VII, VIII, IX & X: Complete *in vitro* testing with CAvitro or preferably a MNvitro. Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test(s) to investigate: - gene mutations *in vivo* if CAvitro or MNvitro is negative (TGR, comet or Pig-a). - both structural or numerical chromosome aberrations and gene mutations if CAvitro or MNvitro is positive (combined MNvivo and comet preferably) If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet (combined or not with MNvivo) is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.			Ensure that all tests together with other available information enable a thorough assessment for gene mutations and effects on chromosome structure and number. Consider on a case-by-case basis the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
14	a	neg	n/a							

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
	b	neg	n/a	neg			Annex VII: no further tests are required. Annexes VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select an adequate *in vivo* somatic cell test to investigate structural or numerical chromosome aberrations (combined MNvivo and comet preferably, or CAvivo or comet for *in vitro* clastogens, MNvivo for *in vitro* aneugens) If necessary, seek expert advice.			Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider on a case-by-case basis the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
	c	n/a	n/a	neg						

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
15		n/a	n/a	n/a			Annexes VII, VIII, IX & X: Check systemic availability before progressing to *in vivo* tests. Select adequate *in vivo* somatic cell test(s) to investigate both structural or numerical chromosome aberrations and gene mutations (combined MNvivo and comet preferably) If the TGR is to be conducted on somatic tissues, germ cell samples must be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells. If the comet (combined or not with MNvivo) is to be conducted on somatic tissues, germ cell sampling is recommended for analysis in case of a positive result in somatic cells. If necessary, seek expert advice.			Ensure that all tests together with other available information enable a thorough assessment for gene mutations and for structural and numerical chromosomal aberrations. Consider on a case-by-case basis the need for further tests to understand the *in vivo* mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
16	a	pos	neg			neg	Annexes VII, VIII, IX & X: no further tests are required.	not genotoxic		
	b	neg	neg	pos		neg				
	c	neg	pos		neg					
	d	n/a	neg	pos		neg				
	e	n/a	neg	n/a		neg				

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
	f	n/a	pos	neg	neg					Further *in vivo* test may be necessary, depending on the quality and relevance of available data. To conclude on the absence of chromosome aberration potential, the Cytvivo study must address the endpoint(s) for which the Cytvitro study was positive, i.e. structural chromosome aberrations or numerical chromosome aberrations, or both.
	g	neg	n/a		neg					
17	a	pos	neg			pos	Annexes VII, VIII, IX & X: No further testing in somatic cells is needed. Annexes VII & VIII: Germ cell mutagenicity investigation should be considered if samples are available. Annexes IX & X: Germ cell mutagenicity testing must be conducted. If necessary, seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the appropriate germ cell mutagenicity test(s).	genotoxic	Expert judgement is needed at this stage to consider whether there is sufficient information to conclude that the substance poses a mutagenic hazard to germ cells. If the data meet the classification conditions for waiving the germ cell mutagenicity information requirements, it can be concluded that the substance causes heritable genetic damage and no further testing is justified.	If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation is necessary, depending on the Annex. Risk assessment and C&L must be completed.
	b	neg	pos		pos					
	c	neg	neg	pos		Pos				
	d	n/a	pos	neg	pos					
	e	n/a	neg	pos		pos				
	f	n/a	neg	n/a		pos				
	g	neg	n/a	neg	pos					
18	a	pos	pos	(pos)	pos			genotoxic		

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
	b	pos	pos	(pos)		pos	Annexes VII, VIII, IX & X: Select adequate *in vivo* somatic cell tests to investigate the remaining endpoint not addressed by the available *in vivo* test. Annexes VII & VIII: Germ cell mutagenicity investigation should be considered if samples are available. Annexes IX & X: Germ cell mutagenicity testing must be conducted. If necessary, seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the appropriate germ cell mutagenicity test(s).		Expert judgement is needed at this stage to consider whether there is sufficient information to conclude that the substance poses a mutagenic hazard to germ cells. If the data meet the classification conditions for waiving the germ cell mutagenicity information requirements, it can be concluded that the substance causes heritable genetic damage and no further testing is justified.	If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation is necessary, depending on the Annex. For instance, if there is concern for both chromosome aberrations and gene mutations but only one endpoint has been clarified in germ cells, the remaining endpoint must be investigated, unless the data already meet the classification conditions for waiving further testing. Risk assessment and C&L must be completed.
19	a	pos	pos	(pos)	neg		Annexes VII, VIII, IX & X: Select adequate *in vivo* somatic cell tests to investigate the remaining endpoint not addressed by the available *in vivo* test. If necessary, seek expert advice.			To conclude on the absence of chromosome aberration potential, the Cytvivo study must address the endpoint(s) for which the Cytvitro study was positive, i.e. structural chromosome aberrations or numerical chromosome aberrations, or both.
	b	pos	pos	(pos)		Neg				

Scenario		Test results (reliably concluded)					Standard information required General follow up procedure	Conclusion	Specific rules for adaptation *[for detailed guidance, incl. timing of the tests, see main text]*	Comments
		GM bact	Cyt *vitro*	GM *vitro*	Cyt *vivo*	GM *vivo*				
20		pos	pos	(pos)	neg	neg	Annexes VII, VIII, IX & X: no further tests are required.	not genotoxic	Further *in vivo* test may be necessary pending on the quality and relevance of available data.	To be considered appropriate, the Cytvivo study must address the endpoint(s) for which the Cytvitro study was positive, i.e. structural chromosome aberrations or numerical chromosome aberrations, or both. Risk assessment and C&L can be completed.
21	a	pos	pos	(pos)	neg	pos	Annexes VII, VIII, IX & X: No further testing in somatic cells is needed. Annexes VII & VIII: Germ cell mutagenicity investigation should be considered if samples are available. Annexes IX & X: Germ cell mutagenicity testing must be conducted (to further investigate the endpoint that is positive in somatic cells). If necessary, seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the appropriate germ cell mutagenicity test(s).	genotoxic	Expert judgement is needed at this stage to consider whether there is sufficient information to conclude that the substance poses a mutagenic hazard to germ cells. If the data meet the classification conditions for waiving the germ cell mutagenicity information requirements, it can be concluded that the substance causes heritable genetic damage and no further testing is justified.	To conclude on the absence of chromosome aberration potential, the Cytvivo study must address the endpoint(s) for which the Cytvitro study was positive, i.e. structural chromosome aberrations or numerical chromosome aberrations, or both. If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation is necessary. Risk assessment and C&L must be completed.
	b	pos	pos	(pos)	pos	neg				

Abbreviations: pos: positive; neg: negative; (pos): the follow up is independent from the result of this test; n/a: the test is not applicable to the substance and/or technically not possible; GM_{bact}: gene mutation test in bacteria (Ames test); Cyt_{vitro}: cytogenetic assay in mammalian cells; CA_{vitro}: *in vitro* chromosome aberration test; MN_{vitro}: *in vitro* micronucleus test; GM_{vitro}: gene mutation assay in mammalian cells; Cyt_{vivo}: somatic cell cytogenetic assay in experimental animals; GM_{vivo}: somatic cell gene mutation assay in experimental animals; CA_{vivo}: *in vivo* chromosome aberration test (bone marrow); MN_{vivo}: *in vivo* micronucleus test (erythrocytes); TGR: *in vivo* gene mutation test with transgenic rodents; comet: *in vivo* comet assay; Pig-a: *in vivo* Pig-a assay.

R.7.7.7 References on mutagenicity

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