

# Guidance on information requirements and chemical safety assessment

## Chapter R.7a: Endpoint specific guidance

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## 1 **R.7.7 Mutagenicity and carcinogenicity**

### 2 **R.7.7.1 Mutagenicity**

#### 3 R.7.7.1.1 Definition of mutagenicity

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

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

11 *Mutagenicity* refers to the induction of permanent transmissible changes in the amount or  
12 structure of the genetic material of cells or organisms. These changes may involve a single  
13 gene or gene segment, a block of genes or chromosomes. The term clastogenicity is used for  
14 agents giving rise to structural chromosome aberrations. A clastogen can cause breaks in  
15 chromosomes that result in the loss or rearrangements of chromosome segments.  
16 Aneugenicity (aneuploidy induction) refers to the effects of agents that give rise to a change  
17 (gain or loss) in chromosome number in cells. An aneugen can cause loss or gain of  
18 chromosomes resulting in cells that have not an exact multiple of the haploid number. For  
19 example, three number 21 chromosomes or trisomy 21 (characteristic of Down syndrome) is a  
20 form of aneuploidy.

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

#### 27 R.7.7.1.2 Objective of the guidance on mutagenicity

28 The aims of testing for genotoxicity are to assess the potential of substances to induce  
29 genotoxic effects which may lead to cancer or cause heritable damage in humans. Genotoxicity  
30 data are used in risk characterisation and classification of substances.

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

35 Mutations in somatic cells may be lethal or may be transferred to daughter cells with  
36 deleterious consequences for the affected organism (*e.g.* cancer may result when they occur in  
37 proto-oncogenes, tumour suppressor genes and/or DNA repair genes) ranging from trivial to  
38 detrimental or lethal.

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

43 Heritable damage to the offspring, and possibly to subsequent generations, of parents exposed  
44 to substances that are mutagens may follow if mutations are induced in parental germ cells. To

1 date, all known germ cell mutagens are also mutagenic in somatic cells *in vivo*. Substances  
2 that are mutagenic in somatic cells may produce heritable effects if they, or their active  
3 metabolites, interact with the genetic material of germ cells. Conversely, substances that do  
4 not induce mutations in somatic cells *in vivo* would not be expected to be germ cell mutagens.

#### 5 **R.7.7.2 Information requirements on mutagenicity**

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

15 Column 1 of the Annexes VII to X of REACH inform on the standard information requirements  
16 for substances produced or imported in quantities of >1 t/y, >10 t/y, >100 t/y, and >1000  
17 t/y, respectively.

18 Column 2 of the Annexes VII-X list specific rules according to which the required standard  
19 information may be omitted, replaced by other information, provided at a different stage or  
20 adapted in another way. If the conditions are met under which column 2 of this Annex allows  
21 adaptations, the fact and the reasons for each adaptation should be clearly indicated in the  
22 registration.

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

1 **Table R.7.7-1 REACH information requirements for mutagenicity**

<b>COLUMN 1 STANDARD INFORMATION REQUIRED</b>	<b>COLUMN 2 SPECIFIC RULES FOR ADAPTATION FROM COLUMN 1</b>
Annex VII: 1. <i>In vitro</i> gene mutation study in bacteria.	Further mutagenicity studies shall be considered in case of a positive result.
Annex VIII: 1. <i>In vitro</i> cytogenicity study in mammalian cells or <i>in vitro</i> micronucleus study.  2. <i>In vitro</i> gene mutation study in mammalian cells, if a negative result in Annex VII, 1 and Annex VIII, 1.	1. The study does not usually need to be conducted <ul style="list-style-type: none"> <li>- if adequate data from an <i>in vivo</i> cytogenicity test are available or</li> <li>- the substance is known to be carcinogenic category 1A or 1B or germ cell mutagenic category 1A, 1B or 2.</li> </ul> 2. The study does not usually need to be conducted if adequate data from a reliable <i>in vivo</i> mammalian gene mutation test are available. Appropriate <i>in vivo</i> mutagenicity studies shall be considered in case of a positive result in any of the genotoxicity studies in Annex VII or VIII.
Annex IX:	If there is a positive result in any of the <i>in vitro</i> genotoxicity studies in Annex VII or VIII and there are no results available from an <i>in vivo</i> study already, an appropriate <i>in vivo</i> somatic cell genotoxicity study shall be proposed by the registrant.  If there is a positive result from an <i>in vivo</i> somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered.
Annex X:	If there is a positive result in any of the <i>in vitro</i> genotoxicity studies in Annex VII or VIII, a second <i>in vivo</i> somatic cell test may be necessary, depending on the quality and relevance of all the available data.  If there is a positive result from an <i>in vivo</i> somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered.

2

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

6 In some cases, the rules set out in Annex VII to XI may require certain tests to be undertaken  
7 earlier than or in addition to the tonnage-triggered requirements. See Section [R.7.7.6](#) for  
8 further guidance on testing requirements.

9 **R.7.7.3 Information and its sources on mutagenicity**

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

1 Typically, *in vitro* tests are performed with cultured bacterial cells, human or other mammalian  
2 cells. The sensitivity and specificity of tests will vary with different classes of substances and, if  
3 adequate data are available for the class of substance to be tested, can guide the selection of  
4 the most appropriate test systems to be used. In order to detect mutagenic effects also of  
5 substances that need to be metabolically activated to become mutagenic, an exogenous  
6 metabolic activation system is usually added in *in vitro* tests. For this purpose the post-  
7 mitochondrial 9000 x *g* supernatant (S-9 fraction) of whole liver tissue homogenate containing  
8 a high concentration of metabolising enzymes is most commonly employed. In the case when  
9 information is required on the mutagenic potential of a substance *in vivo*, several test methods  
10 are available. In *in vivo* tests whole animals are used, in which metabolism and toxicokinetic  
11 mechanisms in general exist as natural components of the test animal. It should be noted that  
12 species-specific differences in metabolism are known. Therefore, different genotoxic responses  
13 may be obtained. Some *in vivo* genotoxicity tests employ methods by which any tissue of an  
14 animal can be examined for effects on the genetic material, giving the possibility to examine  
15 site-of-contact tissues (*i.e.* skin, epithelium of the respiratory or gastro-intestinal tract).

16 Some test methods have an officially adopted EU and/or OECD test guideline (TG) for the  
17 testing procedure, but for some others this is not the case. Furthermore, modifications to  
18 OECD protocols have been developed for various classes of substances and may serve to  
19 enhance the accuracy of test results. Use of such modified protocols is a matter of expert  
20 judgement and will vary as a function of the chemical and physical properties of the substance  
21 to be evaluated.

#### 22 R.7.7.3.1 Non-human data on mutagenicity

##### 23 **Non-testing data on mutagenicity**

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

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

36 *Weight of Evidence* approaches that use expert judgement to include test results for close  
37 chemical analogues are ways of strengthening regulatory positions on the mutagenicity of a  
38 substance. Methods that identify general *structural alerts* for genotoxicity such as the Ashby-  
39 Tennant super-mutagen molecule (Ashby and Tennant, 1988) may also be useful.

##### 41 **Models for mutagenicity**

42 There are hundreds of (Q)SAR models available in the literature for predicting test results for  
43 genotoxic endpoints for closely related structures (Naven *et al.*, 2012; Bakhtyari *et al.*, 2013).  
44 These are known as *local* (Q)SARs. When essential features of the information domain are  
45 clearly represented, these models may constitute the best predictive tools for estimating a  
46 number of mutagenic/genotoxic endpoints. However, quality of reporting varies from model to  
47 model and predictivity must be assessed case-by-case on the basis of clear documentation.  
48 Use of harmonised templates, such as the QSAR Model Reporting Format (QMRF) and the  
49 QSAR Prediction Reporting Format (QPRF) developed by the the European Commission Joint

1 Research Centre ([http://ihcp.jrc.ec.europa.eu/our\\_labs/predictive\\_toxicology/qsar\\_tools/QRf](http://ihcp.jrc.ec.europa.eu/our_labs/predictive_toxicology/qsar_tools/QRf)),  
2 can help ensure consistency in summarising and reporting key information on (Q)SAR models  
3 and substance-specific predictions generated by (Q)SAR models.

4 Generally, (Q)SAR models that contain putative mechanistic descriptors are preferred;  
5 however many models use purely structural descriptors. While such models may be highly  
6 predictive, they rely on statistical methods and the toxicological significance of the descriptors  
7 may be obscure.

8 Another type of (Q)SAR model for mutagenicity attempts to predict (within their domain)  
9 diverse (non-congeneric) groups of substances. These are termed *global* (Q)SARs and are far  
10 more ambitious than the more simple local models. Global (Q)SARs are usually implemented in  
11 computer programs, some of which first divide chemicals into local (Q)SARs, i.e. categorise the  
12 input molecule into the chemical domain it belongs to, and then apply the corresponding  
13 prediction model. These are known as expert systems. Other global models apply the same  
14 mathematical algorithm on all input molecules without prior separation. It is generally  
15 observed that the concept of applicability domain is a useful one and the endpoints for  
16 chemicals inside the applicability domains of the models are better predicted than for  
17 chemicals falling outside.

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

25 The most common genotoxicity endpoint for global models has been to predict results of the  
26 Ames test. Some models for this endpoint include a metabolic simulator.

27 There are models for many other mutagenicity endpoints. For example, the Danish EPA and  
28 the Danish QSAR group at DTU Food (National Food Institute at the Technical University of  
29 Denmark) have developed a (Q)SAR database that contains predictions from a number of  
30 mutagenicity models. In addition to assorted Ames models, the database contains predictions  
31 of the following *in vitro* endpoints: chromosomal aberrations (CHO and CHL cells), mouse  
32 lymphoma/*tk*, CHO/hprt gene-mutation assays and UDS (rat hepatocytes); and the following  
33 *in vivo* endpoints: *Drosophila* SLRL, mouse micronucleus, rodent dominant lethal, mouse SCE  
34 in bone marrow and mouse Comet assay data. The database is freely accessible via  
35 <http://qsar.food.dtu.dk>. The online database contains predictions for over 166,000 chemicals  
36 and includes a flexible system for chemical structure and parameter searching. A user manual  
37 with information on the individual models including training set information and validation  
38 results is available at the website. The database is also integrated into the OECD (Q)SAR  
39 Toolbox. A major update of the database with consensus predictions by use of different QSAR  
40 models for each of the modelled endpoints for more than 600,000 structures, including over  
41 70,000 REACH pre-registered substances, and with an improved user interface is scheduled for  
42 the beginning of 2015.

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

49 Neither of these two examples is perfect, but they illustrate a trend towards predictions of  
50 multiple endpoints and may assist those making *Weight of Evidence* decisions regarding the  
51 mutagenic potential of untested substances. More detailed information on the strengths and  
52 limitations of the different (Q)SAR models can be found elsewhere (Serafimova *et al.*, 2010).

1

## 2 Databases with experimental data

3 There are several open-source databases with experimental information on mutagenicity and  
4 carcinogenicity (the two endpoints can often not easily be separated). A review of these  
5 databases can be found in Serafimova *et al.* (2010).

6

## 7 OECD QSAR Toolbox

8 To increase the regulatory acceptance of (Q)SAR models, the OECD has started the  
9 development of a QSAR Toolbox to make (Q)SAR technology readily accessible, transparent  
10 and less demanding in terms of infrastructure costs (<http://www.qsartoolbox.org/>). The OECD  
11 QSAR Toolbox facilitates the practical application of grouping and read-across approaches to fill  
12 gaps in (eco-)toxicity data, including genotoxicity and genotoxic carcinogenicity, for chemical  
13 hazard assessment. In particular, the OECD QSAR Toolbox covers the *in vitro* gene mutation  
14 (Ames test), *in vitro* chromosomal aberration, *in vivo* chromosomal aberration (micronucleus  
15 test), and genotoxic carcinogenicity endpoints. The predictions are based on the  
16 implementation of a range of profilers connected with genotoxicity and carcinogenicity (to  
17 quickly evaluate chemicals for common mechanisms or modes of action), and the incorporation  
18 of numerous databases with results from experimental studies (to support read-across and  
19 trend analysis) into a logical workflow. The Toolbox and guidance on its use are freely  
20 available.

21

22 The *Guidance on IR&CSA Chapter R.6: QSARs and grouping of chemicals* explains basic  
23 concepts of (Q)SARs and gives generic guidance on validation, adequacy and documentation  
24 for regulatory purposes. It also describes a stepwise approach for the use of read-  
25 across/grouping and (Q)SARs. Further information on the category formation and read-across  
26 approach for the prediction of toxicity can be found in Enoch (2010).

27

28

1 Testing data on mutagenicity  
 2 Test methods preferred for use are listed below. Some of these have officially adopted EU (see  
 3 Regulation (EC) No 440/2008) and/or OECD  
 4 (<http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>) guidelines,  
 5 the others are regarded as scientifically acceptable for genotoxicity testing.

6 *In vitro* data

7 **Table R.7.7-2 *In vitro* test methods**

Test method	GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD	EU/OECD guideline
Bacterial reverse mutation test	Gene mutations/The test uses amino-acid requiring strains of bacteria to detect (reverse) gene mutations (point mutations and frameshifts).	EU: B.13/14 OECD: 471
<i>In vitro</i> mammalian cell gene mutation test – <i>hprt</i> test	Gene mutations/The test identifies chemicals that induce gene mutations in the <i>hprt</i> gene of established cell lines.	EU: B.17 OECD: 476
<i>In vitro</i> mammalian cell gene mutation test – Mouse lymphoma assay	Gene mutations and structural chromosome aberrations/The test identifies chemicals that induce gene mutations in the <i>tk</i> gene of the L5178Y mouse lymphoma cell line. If colonies in a <i>tk</i> mutation test are scored using the criteria of normal growth (large) and slow growth (small) colonies, gross structural chromosome aberrations may be measured, since mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and are more likely to form small colonies.	EU: B.17 OECD: 476
<i>In vitro</i> mammalian chromosome aberration test	Structural and numerical chromosome aberrations/The test identifies chemicals that induce chromosome aberrations in cultured mammalian established cell lines, cell strains or primary cell cultures. An increase in polyploidy may indicate that a chemical has the potential to induce numerical chromosome aberrations.	EU: B.10 OECD: 473
<i>In vitro</i> micronucleus test	Structural and numerical chromosome aberrations/The test identifies chemicals 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 chemicals.	EU: none OECD: 487

8

9 As noted earlier, accepted modifications to the standard test protocols have been developed to  
 10 enhance test sensitivity to specific classes of substances. Expert judgement should be applied  
 11 to judge whether any of these are appropriate for a given substance being registered. For  
 12 example, protocol modifications for the Ames assay might be appropriate for substances such  
 13 as gases, volatile liquids, azo-dyes, diazo compounds, glycosides, and petroleum oil derived  
 14 products, which should be regarded as special cases.

15 *Animal data*

16 *Somatic cells*

1 **Table R.7.7-3 *In vivo* test methods, somatic cells**

<b>Test method</b>	<b>GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD</b>	<b>EU/OECD guideline</b>
<i>In vivo</i> mammalian bone marrow chromosome aberration test	Structural and numerical chromosome aberrations/The test identifies chemicals that induce chromosome aberrations in the bone-marrow cells of animals, usually rodents. An increase in polyploidy may indicate that a chemical has the potential to induce numerical chromosome aberrations.	EU: B.11 OECD: 475
<i>In vivo</i> mammalian erythrocyte micronucleus test	Structural and numerical chromosome aberrations/The test identifies chemicals that cause micronuclei in erythroblasts sampled from bone marrow and/or peripheral blood cells of animals, usually rodents. These micronuclei may originate from acentric fragments or whole chromosomes, and the test thus has the potential to detect both clastogenic and aneugenic chemicals.	EU: B.12 OECD: 474
Unscheduled DNA synthesis (UDS) test with mammalian liver cells <i>in vivo</i>	DNA repair/The test identifies chemicals that induce 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.	EU: B.39 OECD: 486
Transgenic rodent (TGR) somatic cell mutation assays	Gene mutations/The tests can measure gene mutations in any tissue of an animal and may, therefore, also be used in specific site of contact tissues.	EU: none OECD: 488
<i>In vivo</i> alkaline single-cell gel electrophoresis assay for DNA strand breaks (Comet assay)	DNA strand breaks/The test can measure DNA strand breaks in any tissue of an animal and may, therefore, also be used in specific site of contact tissues.	EU: none OECD: none

2

3 A detailed review of transgenic animal model assays, including recommendations on the  
4 conduct of such assays in somatic cells, has been produced for the OECD (Lambert *et al.*,  
5 2005; OECD, 2009).

6 Protocols and recommendations for conducting the *in vivo* alkaline single-cell gel  
7 electrophoresis assay for DNA strand breaks (Comet assay) have been published by various  
8 international groups (Tice *et al.*, 2000; Hartmann *et al.*, 2003; McKelvey-Martin *et al.*, 1993;  
9 Brendler-Schwaab *et al.*, 2005; Burlinson *et al.*, 2007; Smith *et al.*, 2008; Rothfuss *et al.*,  
10 2010; Burlinson, 2012; Vasquez, 2012; Johansson *et al.*, 2010; Kirkland & Speit, 2008; EFSA,  
11 2012). An international validation study on the *in vivo* alkaline single-cell gel electrophoresis  
12 assay was coordinated by the Japanese Centre for the Validation of Alternative Methods  
13 (JaCVAM) from 2006 to 2012. The validation study report was peer reviewed by the OECD and  
14 an OECD expert group is currently working on the drafting of the TG, with a target date for  
15 adoption by the OECD Working Group of National Coordinators of the Test Guidelines  
16 Programme (WNT) in April 2014.

17

18 **Germ cells**

19 Testing in germ cells will be conducted only on very rare occasions (see Section [R.7.7.6](#)).

1 **Table R.7.7-4 *In vivo* test methods, germ cells**

Test method	GENOTOXIC ENDPOINTS measured/ PRINCIPLE OF THE TEST METHOD	EU/OECD guideline
Mammalian spermatogonial chromosome aberration test	Structural and numerical chromosome aberrations/The test measures 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 chemical has the potential to induce numerical chromosome aberrations.	EU: B.23 OECD: 483
Rodent dominant lethal test	Structural and numerical chromosome aberrations/The test measures 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 lethals are due to structural and numerical chromosome aberrations. Rats or mice are recommended as the test species.	EU: B.22 OECD: 478
Transgenic rodent (TGR) germ cell mutation assays	Gene mutations/ The tests measure gene mutations in spermatocytes of an animal and may, therefore, be used to obtain information about the mutagenic activity of a chemical in germ cells.	EU: none OECD: 488
<i>In vivo</i> alkaline single-cell gel electrophoresis assay for DNA strand breaks (Comet assay)	DNA strand breaks/ The test measures DNA strand breaks in spermatocytes of an animal and may, therefore, be used to obtain information about the DNA-damaging activity of a chemical in germ cells.	EU: none OECD: none

2

3 A detailed review of transgenic animal model assays, including recommendations on the  
4 conduct of such assays in germ cells, has been produced for the OECD (Lambert *et al.*, 2005;  
5 OECD, 2009).

#### 6 R.7.7.3.2 Human data on mutagenicity

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

#### 11 **R.7.7.4 Evaluation of available information on mutagenicity**

12 Genotoxicity is a complex endpoint and requires evaluation by expert judgement. For both  
13 steps of the effects assessment, *i.e.* hazard identification and dose (concentration)-response  
14 (effect) assessment, it is very important to evaluate the data with regard to their adequacy  
15 and completeness. The evaluation of adequacy should address the reliability and relevance of  
16 the data in a way as outlined in the introductory chapter. The completeness of the data refers  
17 to the conclusion on the comparison between the available adequate information and the  
18 information that is required under the REACH proposal for the applicable tonnage level of the  
19 substance. Such a conclusion relies on *Weight of Evidence* approaches, mentioned in Annex XI  
20 Section 1.2 of REACH, which categorise available information based on the methods used:  
21 *guideline tests*, *non-guideline tests*, and other types of information which may justify  
22 adaptation of the standard testing regime. Such a *Weight of Evidence* approach also includes  
23 an evaluation of the available data as a whole, *i.e.* both *over and across* toxicological  
24 endpoints.

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

#### 1 R.7.7.4.1 Non-human data on mutagenicity

##### 2 **Non-testing data for mutagenicity**

3 In a more formal approach, documentation can include reference to a related chemical or  
4 group of chemicals that leads to the conclusion of concern or lack of concern. This can either  
5 be presented according to scientific logic (read-across) or sometimes as a mathematical  
6 relationship of chemical similarity.

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

13 Chemicals for which no test-data exist present a special case in which reliance on non-testing  
14 data may be absolute. Many factors will dictate the acceptability of non-testing methods in  
15 reaching a conclusion based on no tests at all. It is yet to be established whether *Weight of*  
16 *Evidence* decisions based on multiple genotoxicity and carcinogenicity estimates can equal or  
17 exceed those obtained by one or two *in vitro* tests. This must be considered on a case-by-case  
18 basis.

##### 19 **Testing data on mutagenicity**

20 Evaluation of genotoxicity test data should be made with care. Regarding *positive* findings,  
21 responses generated only at highly toxic/cytotoxic concentrations should be interpreted with  
22 caution, and the presence or absence of a dose-response relationship should be considered.

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

- 24 • the doses or concentrations of test substance used (were they high enough?)
- 25 • was the test system used sensitive to the nature of the genotoxic changes that might  
26 have been expected? For example, some *in vitro* test systems will be sensitive to point  
27 mutations and small deletions but not to mutagenic events that create large deletions.
- 28 • the volatility of the test substance (were concentrations maintained in tests conducted  
29 *in vitro*?)
- 30 • for studies *in vitro*, the possibility of metabolism not being active in the system  
31 including those in extra-hepatic organs
- 32 • was the test substance taken up by the test system used for *in vitro* studies?
- 33 • for studies *in vivo*, is the substance reaching the target organ? (taking also toxicokinetic  
34 data into consideration, e.g. rate of hydrolysis and electrophilicity may be factors that  
35 need to be considered)

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

- 38 • conflicting results obtained in non-mammalian systems and in mammalian cell tests  
39 may be addressed by considering possible differences in substance uptake, metabolism  
40 or in the organisation of genetic material. Although the results of mammalian tests may  
41 be considered of higher significance, additional data may be needed to resolve  
42 contradictions
- 43 • if the results of indicator tests (e.g. DNA binding, DNA damage, DNA repair; SCE) are  
44 not supported by results obtained in tests for mutagenicity, the results of mutagenicity  
45 tests are generally of higher significance

- 1 • if contradictory findings are obtained *in vitro* and *in vivo*, in general, the results of *in*  
2 *vivo* tests indicate a higher degree of reliability. However, for evaluation of *negative*  
3 results *in vivo*, it should be considered whether there is adequate evidence of target  
4 tissue exposure
- 5 • the sensitivity and specificity of different test systems varies for different classes of  
6 substances. If available testing data for other related substances permits assessment of  
7 the performance of difference assays for the class of substance under evaluation, the  
8 result from the test system known to produce more accurate responses would be given  
9 higher priority

10 Conflicting results may be also available from the same test, performed by different  
11 laboratories or on different occasions. In this case, expert judgement should be used to reach  
12 an overall evaluation of the data. In particular, the quality of each of the studies and of the  
13 data provided should be evaluated, with special consideration of the study design,  
14 reproducibility of data, dose-effect relationships, and biological relevance of the findings. The  
15 purity of the test substance may also be a factor to take into account. In the case where an  
16 EU/OECD guideline is available for a test method, the quality of a study using the method is  
17 regarded as being higher if it was conducted in compliance with the requirements stated in the  
18 guideline. Furthermore, studies compliant with GLP may be regarded as being of a higher  
19 quality.

20 When making an assessment of the potential mutagenicity of a substance, or considering the  
21 need for further testing, data from various tests and genotoxic endpoints may be found. Both  
22 the strength and the weight of the evidence should be taken into account. The strongest  
23 evidence will be provided by modern, well-conducted studies with internationally established  
24 test protocols. For each test type and each genotoxic endpoint, there should be a separate  
25 *Weight of Evidence* analysis. It is not unusual for positive evidence of mutagenicity to be found  
26 in just one test type or for only one endpoint. In such cases the positive and negative results  
27 for different endpoints are not conflicting, but illustrate the advantage of using test methods  
28 for a variety of genetic alterations to increase the probability of identifying substances with  
29 mutagenic potential. Hence, results from methods testing different genotoxic endpoints should  
30 not be combined in an overall *Weight of Evidence* analysis, but should be subjected to such  
31 analysis separately.

#### 32 R.7.7.4.2 Human data on mutagenicity

33 Human data have to be assessed carefully on a case-by-case basis. The interpretation of such  
34 data requires considerable expertise. Attention should be paid especially to the adequacy of  
35 the exposure information, confounding factors, co-exposures and to sources of bias in the  
36 study design or incident. The statistical power of the test may also be considered.

#### 37 R.7.7.4.3 Remaining uncertainty on mutagenicity

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

### 41 **R.7.7.5 Conclusions on mutagenicity**

#### 42 R.7.7.5.1 Concluding on suitability for Classification and Labelling

43 In order to conclude on an appropriate classification and labelling position with regard to  
44 mutagenicity, the available data should be considered using the criteria according to Annex I  
45 to the CLP Regulation (EC) No 1272/2008.

## 1 R.7.7.5.2 Concluding on suitability for Chemical Safety Assessment

### 2 **Considerations on dose response shapes and mode of action of mutagenic** 3 **substances in test systems**

4 Considerations of the dose-response relationship and of possible mechanisms of action are  
5 important components of a risk assessment. The default assumption for genotoxic chemicals,  
6 in the absence of mechanistic evidence to the contrary, is that they have a linear dose-  
7 response relationship. However, both direct and indirect mechanisms of genotoxicity can be  
8 non-linear or thresholded and, consequently, sometimes this default assumption may be  
9 inappropriate.

10 Examples of mechanisms of genotoxicity that may be demonstrated to lead to non-linear or  
11 thresholded dose-response relationships include extremes of pH, ionic strength and osmolarity,  
12 inhibition of DNA synthesis, alterations in DNA repair, overloading of defence mechanisms  
13 (anti-oxidants or metal homeostatic controls), interaction with microtubule assembly leading to  
14 aneuploidy, topoisomerase inhibition, high cytotoxicity, metabolic overload and physiological  
15 perturbations (*e.g.* induction of erythropoiesis). Assessment of the significance to be assigned  
16 to genotoxic responses mediated by such mechanisms would include an assessment of  
17 whether the underlying mechanism can be induced at substance concentrations that can be  
18 expected to occur under relevant *in vivo* conditions.

19 In general, several doses are tested in genotoxicity assays. Determination of experimental  
20 dose-effect relationships may be used to assess the genotoxic potential of a substance, as  
21 indicated below. It should be recognised that not all of these considerations may be applicable  
22 to *in vivo* data.

- 23 • a dose-related increase in genotoxicity is one of the relevant criteria for identification of  
24 positive findings. In practice, this will be most helpful for *in vitro* tests, but care is  
25 needed to check for cytotoxicity or cell cycle delay which may cause deviations from a  
26 dose-response related effect in some experimental systems
- 27 • genotoxicity tests are not designed in order to derive no effect levels. However, the  
28 magnitude of the lowest dose with an observed effect (*i.e.* the Lowest Observed Effect  
29 Dose or LOED) may, on certain occasions, be a helpful tool in risk assessment. This is  
30 true specifically for genotoxic effects caused by thresholded mechanisms, like, *e.g.*  
31 aneugenicity. Further, it can give an indication of the mutagenic potency of the  
32 substance in the test at issue. Modified studies, with additional dose points and  
33 improved statistical power may be useful in this regard
- 34 • unusual shapes of dose-response curves may contribute to the identification of specific  
35 mechanisms of genotoxicity. For example, extremely steep increases suggest an  
36 indirect mode of action or metabolic switching which could be confirmed by further  
37 investigation

### 38 **Considerations on genetic risks associated with human exposure to** 39 **mutagenic substances**

40 There are no officially adopted methods for estimating health risks associated with (low)  
41 exposures of humans to mutagens. In fact, most – if not all tests used today – are developed  
42 and applied to identify mutagenic properties of the substance, *i.e.* identification of the  
43 mutagenic hazard *per se*. In today's regulatory practice, the assessment of human health risks  
44 from exposure to mutagenic substances is considered to be covered by assessing and  
45 regulating the carcinogenic risks of these agents. The reason for this is that mutagenic events  
46 underlie these carcinogenic effects. Therefore, mutagenicity data is not used for deriving dose  
47 descriptors for risk assessment purposes and the reader is referred to this aspect in Section  
48 R.7.7.8 (Carcinogenicity) for guidance on how to assess the chemical safety for mutagenic  
49 substances.

### 1 R.7.7.5.3 Information not adequate

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

## 6 **R.7.7.6 Integrated Testing Strategy (ITS) for mutagenicity**

### 7 R.7.7.6.1 Objective / General principles

8 This testing strategy describes a flexible, stepwise approach for hazard identification with  
9 regard to the mutagenic potential of substances, so that sufficient data may be obtained for  
10 adequate risk characterisation including classification and labelling. It serves to help minimise  
11 the use of animals and costs as far as is consistent with scientific rigour. A flow chart and a  
12 summary of the testing strategy are presented in Figure R.7.7-1 and Figure R.7.7-12,  
13 respectively. As noted later in this section, deviations from this strategy may be considered if  
14 existing data for related substances indicate that alternate testing strategies yield results with  
15 greater sensitivity and specificity for mutagenicity *in vivo*.

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

21 For some substances, relevant data from other sources/tests may also be available (*e.g.*  
22 physico-chemical, toxicokinetic, and toxicodynamic parameters and other toxicity data; data  
23 on well-investigated, structurally similar, chemicals). These should be reviewed because,  
24 sometimes, they may indicate that either more or less genotoxicity studies are needed on the  
25 substance than defined by standard information requirements; *i.e.* they may allow tailored  
26 testing/selection of test systems. For example, bacterial mutagenesis assays of inorganic metal  
27 compounds are frequently negative due to limited capacity for uptake of metal ions. The high  
28 prevalence of false negatives for metal compounds might suggest that mutagenesis assays  
29 with mammalian cells, as opposed to bacterial cells, would be the preferred starting point for  
30 testing for this class of Annex VII substances.

31 In summary, a key concept of the strategy is that initial genotoxicity tests and testing  
32 protocols should be selected with due consideration to pre-existing data that has established  
33 the most accurate testing strategy for the class of compound under evaluation. Even then,  
34 initial testing may not always give adequate information and further testing may sometimes be  
35 considered necessary in the light of all available relevant information on the substance,  
36 including its use pattern. Further testing will normally be required for substances which give  
37 rise to positive results in any of the *in vitro* tests.

38 If negative results are available from an adequate evaluation of genotoxicity from existing data  
39 in appropriate test systems, there may be no requirement to conduct additional genotoxicity  
40 tests.

41 Substances for which there is a formal agreement to classify them in category 1A, 1B or 2 for  
42 mutagenicity and/or category 1A or 1B for carcinogenicity will usually not require additional  
43 testing in order to meet the requirements of Annexes VII-X. In cases where a registrant is  
44 unsure of the formal position on the classification of a substance, or wishes to make a  
45 classification proposal themselves, advice should be sought from an appropriate regulatory  
46 body before proceeding with any further testing.

### 47 R.7.7.6.2 Preliminary considerations

1 For a comprehensive coverage of the potential mutagenicity of a substance, information on  
2 gene mutations (base substitutions and deletions/additions), structural chromosome  
3 aberrations (breaks and rearrangements) and numerical chromosome aberrations (loss or gain  
4 of chromosomes, defined as aneuploidy) is required. This may be obtained from available data  
5 or tests on the substance itself or, sometimes, by prediction using appropriate *in silico*  
6 techniques (e.g. chemical grouping, read-across or (Q)SAR approaches).

7 It is important that whatever is known of the physico-chemical properties of the test substance  
8 is taken into account before devising an appropriate testing strategy. Such information may  
9 impact upon both the selection of test systems to be employed and/or modifications to the test  
10 protocols used. The chemical structure of a substance can provide information for an initial  
11 assessment of mutagenic potential. The need for special testing requirements in relation to  
12 photomutagenicity may be indicated by the structure of a molecule, its light absorbing  
13 potential or its potential to be photoactivated. By using expert judgement, it may be possible  
14 to identify whether a substance, or a potential metabolite of a substance, shares structural  
15 characteristics with known mutagens or non-mutagens. This can be used to justify a higher or  
16 lower level of priority for the characterisation of the mutagenic potential of a substance. Where  
17 the level of evidence for mutagenicity is particularly strong, it may be possible to make a  
18 conclusive hazard assessment in accordance with Annex I of REACH without additional testing  
19 on the basis of structure-activity relationships alone.

20 *In vitro* tests are particularly useful for gaining an understanding of the potential mutagenicity  
21 of a substance and they have a critical role in this testing strategy. They are not, however,  
22 without their limitations. Animal tests will, in general, be needed for the clarification of the  
23 relevance of positive findings and in case of specific metabolic pathways that cannot be  
24 simulated adequately *in vitro*.

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

31 Certain substances in addition to those already noted may need special consideration, such as  
32 highly electrophilic substances that give positive results *in vitro*, particularly in the absence of  
33 metabolic activation. Although these substances may react with proteins and water *in vivo* and  
34 thus be rendered inactive towards many tissues, they may be able to express their mutagenic  
35 potential at the initial site of contact with the body. Consequently, the use of test methods that  
36 can be applied to the respiratory tract, upper gastrointestinal tract and skin may be  
37 appropriate. It is possible that specialised test methods will need to be applied in these  
38 circumstances, and that these may not have recognised, internationally valid, test guidelines.  
39 The validity and utility of such tests and the selection of protocols should be assessed by  
40 appropriate experts or authorities on a case-by-case basis.

41 A substance giving an equivocal test result should be reinvestigated immediately, normally  
42 using the same test method, but varying the conditions to obtain conclusive results. Wherever  
43 possible, clear results should be obtained for one step in the strategic procedure before going  
44 on to the next. In cases where this does not prove to be possible, a further test should be  
45 conducted in accord with the strategy.

46 Tests need not be performed if it is not technically possible to do so, or if they are not  
47 considered necessary in the light of current scientific knowledge. Scientific justifications for not  
48 performing tests required by the strategy should always be documented. It is preferred that  
49 tests as described in OECD Guidelines or Regulation (EC) No 440/2008 are used where  
50 possible. Alternatively, for other tests, up-to-date protocols defined by internationally  
51 recognised groups of experts, e.g. International Workshop on Genotoxicity Testing (IWGT,  
52 under the umbrella of the International Association of Environmental Mutagen Societies), may  
53 be used provided that the tests are scientifically justified. It is essential that all tests be

1 conducted according to rigorous protocols in order to maximise the potential for detecting a  
2 mutagenic response, to ensure that negative results can be accepted with confidence and that  
3 results are comparable when tests are conducted in different laboratories. At the time of  
4 writing this guidance, regulatory guidelines are still to be established for an *in vivo* test  
5 included in the testing strategy described below, *i.e.* the Comet assay. If one of these tests is  
6 to be conducted, consultation on the protocol with an appropriate expert or authority is  
7 advisable.

8 If a registrant wishes to undertake any tests for substances at the Annex IX or X tonnage  
9 levels that require the use of vertebrate animals, then there is a need to make a testing  
10 proposal to the European Chemicals Agency first. Testing may only be undertaken when an  
11 agreement has been reached with the Agency.

### 12 R.7.7.6.3 Testing strategy for mutagenicity

#### 13 **Standard information requirement at Annex VII**

14 A preliminary assessment of mutagenicity is required for substances at the REACH Annex VII  
15 tonnage level. All available information should be included but, as a minimum, there should  
16 normally be data from a gene mutation test in bacteria unless existing data for analogous  
17 substances indicates this would be inappropriate. For substances with significant toxicity to  
18 bacteria, not taken up by bacteria, or for which the gene mutation test in bacteria cannot be  
19 performed adequately, an *in vitro* mammalian cell gene mutation test may be used as an  
20 alternative test.

21 When the result of the bacterial test is positive, it is important to consider the possibility of the  
22 substance being genotoxic in mammalian cells. The need for further test data to clarify this  
23 possibility at the Annex VII tonnage level will depend on an evaluation of all the available  
24 information relating to the genotoxicity of the substance.

#### 25 **Standard information requirement at Annex VIII**

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

29 In order to ensure the necessary minimum level of information is provided, at least one further  
30 test is required in addition to the gene mutation test in bacteria. This should be an *in vitro*  
31 mammalian cell test capable of detecting both structural and numerical chromosome  
32 aberrations.

33 There are essentially two different methods that can be viewed as options for this first  
34 mammalian cell test.

- 35 • An *in vitro* chromosome aberration test (OECD TG 473), *i.e.* a cytogenetic assay for  
36 structural chromosome aberrations using metaphase analysis. It may be possible to  
37 present some preliminary information from this test on potential aneugenicity by  
38 recording the incidence of hyperdiploidy and polyploidy. If this preliminary information  
39 presents a possible concern about aneugenicity, this indicates the need for specific  
40 investigations to assess potential aneugenicity of the chemical. An alternative option  
41 would be to conduct an *in vitro* micronucleus test.
- 42 • An *in vitro* micronucleus test (OECD TG 487). This is a cytogenetic assay that has the  
43 advantage of detecting not only structural chromosomal aberrations but also  
44 aneuploidy. Use of fluorescence *in situ* hybridisation with probes for centromeric DNA or  
45 stains for kinetochore proteins can enable aneugens to be distinguished from  
46 clastogens. This may sometimes be useful for risk characterisation.

1 Other *in vitro* tests may be acceptable as the first mammalian cell test, but care should be  
2 taken to evaluate their suitability for the substance being registered and their reliability as a  
3 screen for chemicals that cause structural and/or numerical chromosome aberrations. A  
4 supporting rationale should be presented for a registration with any of these other tests.

5 It is possible to present existing data from an *in vivo* cytogenetic test (*i.e.* a study or studies  
6 conducted previously) as an alternative to the first *in vitro* mammalian cell test. For instance, if  
7 an adequately performed *in vivo* micronucleus test is available already it may be presented as  
8 an alternative.

9 An *in vitro* gene mutation study in mammalian cells (OECD TG 476) is the second part of the  
10 standard information set required for registration at the Annex VIII tonnage level. For  
11 substances that have been tested already, this information should always be presented as part  
12 of the overall *Weight of Evidence* for mutagenicity. For other substances, this second *in vitro*  
13 mammalian cell test will normally only be required when the results of the bacterial gene  
14 mutation test and the first study in mammalian cells (*i.e.* an *in vitro* chromosome aberration  
15 test or an *in vitro* micronucleus test) are negative. This is to detect *in vitro* mutagens that give  
16 negative results in the other two tests.

17 Under specific circumstances it may be possible to omit the second *in vitro* study in  
18 mammalian cells, *i.e.* if it can be demonstrated that this mammalian cell test will not provide  
19 any further useful information about the potential *in vivo* mutagenicity of a substance, then it  
20 does not need to be conducted. This should be evaluated on a case-by-case basis as there may  
21 be classes of compound for which conclusive data can be provided to show that the sensitivity  
22 of the first two *in vitro* tests cannot be improved by the conduct of the third test.

23 The *in vitro* mammalian cell gene mutation test will not usually be required if adequate  
24 information is available from a reliable *in vivo* study capable of detecting gene mutations. Such  
25 information may come from a TGR gene mutation assay. A Comet assay or a liver UDS test  
26 may also be adequate, however, those tests being indicator assays detecting putative DNA  
27 lesions, their use should be justified on a case-by-case basis.

28 Provided the *in vitro* tests have given negative results, normally, no *in vivo* tests will be  
29 required to fulfil the standard information requirements at Annex VIII. However, there may be  
30 rare occasions when it is appropriate to conduct testing *in vivo*, for example when it is not  
31 possible technically to perform satisfactory tests *in vitro*. Substances which, by virtue of, for  
32 example, their physico-chemical characteristics, chemical reactivity or toxicity cannot be tested  
33 in one or more of the *in vitro* tests should be considered on a case-by-case basis.

## 34 **Requirement for testing beyond the standard levels specified for Annexes VII** 35 **and VIII**

### 36 [Introductory comments](#)

37 Concerns raised by positive results from *in vitro* tests may require the consideration of further  
38 testing. The chemistry of the substance, data on analogous substances, toxicokinetic and  
39 toxicodynamic data, and other toxicity data will also influence the timing and pattern of further  
40 testing.

41 Testing beyond the standard set of *in vitro* tests is first directed towards investigating the  
42 potential for mutagenicity in somatic cells *in vivo*. Positive results in somatic cells *in vivo*  
43 constitute the trigger for consideration of investigation of potential expression of genotoxicity  
44 in germ cells.

45

### 46 [Substances that are negative in the standard set of \*in vitro\* tests](#)

47 In general, substances that are negative in the full set of *in vitro* tests specified in REACH  
48 Annexes VII and VIII are considered to be non-genotoxic. There are only a very limited

1 number of chemicals that have been found to be genotoxic *in vivo*, but not in the standard *in*  
2 *vitro* tests. Most of these are pharmaceuticals designed to affect pathways of cellular  
3 regulation, including cell cycle regulation, and this evidence is judged insufficient to justify  
4 routine *in vivo* testing of industrial chemicals. However, occasionally, knowledge about the  
5 metabolic profile of a substance may indicate that the standard *in vitro* tests are not  
6 sufficiently reassuring and a further *in vitro* test, or an *in vivo* test, may be needed in order to  
7 ensure mutagenicity potential is adequately explored (e.g. use of an alternative to rat liver S-9  
8 mix, a reducing system, a metabolically active cell line like HepG2 cells or genetically  
9 engineered cell lines might be judged appropriate).

#### 10 Substances for which an *in vitro* test is positive

11 REACH Annex VII substances for which only a bacterial gene mutation test has been conducted  
12 and for which the result is positive should be studied further, according to the requirements of  
13 Annex VIII.

14 Regarding Annex VIII, when both the mammalian cell tests are negative but there was a  
15 positive result in the bacterial test, it will be necessary to decide whether any further testing is  
16 needed on a case-by-case basis. For example, suspicion that a unique positive response  
17 observed in the bacterial test was due to a specific bacterial metabolism of the test substance  
18 could be explored further by investigation *in vitro*. Alternatively, an *in vivo* test may be  
19 required (see below).

20 In REACH Annex VIII, following a positive result in an *in vitro* mammalian cell mutagenicity  
21 test, adequately conducted somatic cell *in vivo* testing is required to ascertain if this potential  
22 can be expressed *in vivo*. In cases where it can be sufficiently deduced that a positive *in vitro*  
23 finding is not relevant for *in vivo* situations (e.g. due to the effect of the test substances on pH  
24 or cell viability: see also Section [R.7.7.4.1](#)), *in vivo* testing will not be necessary.

25 Annex VIII, Column 2 requires the registrant to consider appropriate mutagenicity *in vivo*  
26 studies already at the Annex VIII tonnage level, in cases where positive results in genotoxicity  
27 studies have been obtained. It should be noted that where this involves tests mentioned in  
28 Annexes IX or X, such as *in vivo* somatic cell genotoxicity studies, testing proposals must be  
29 submitted by the registrant and accepted by ECHA before testing can be initiated.

30 At Annexes IX and X, if there is a positive result in any of the *in vitro* studies from Annex VII  
31 or VIII and there are no appropriate results available from an *in vivo* study already, an  
32 appropriate *in vivo* somatic cell genotoxicity study should be proposed.

33 Before any decisions are made about the need for *in vivo* testing, a review of the *in vitro* test  
34 results and all available information on the toxicokinetic and toxicodynamic profile of the test  
35 substance is needed. A particular *in vivo* test should be conducted only when it can be  
36 reasonably expected from all the properties of the test substance and the proposed test  
37 protocol that the specific target tissue will be adequately exposed to the test substance and/or  
38 its metabolites. If necessary, a targeted investigation of toxicokinetics should be conducted  
39 before progressing to *in vivo* testing (e.g. a preliminary toxicity test to confirm that absorption  
40 occurs and that an appropriate dose route is used).

41 In the interest of ensuring that the number of animals used in genotoxicity tests is kept to a  
42 minimum, both males and females should not automatically be used. In accord with standard  
43 guidelines, testing in one sex only is possible when the substance has been investigated for  
44 general toxicity and no sex-specific differences in toxicity have been observed. If the test is  
45 performed in a laboratory with substantial experience and historical data, it should be  
46 considered whether a concurrent positive control and a concurrent negative control for all time  
47 points (e.g. for both the 24h and 48h time point in the micronucleus assay) will really be  
48 necessary (Hayashi *et al.*, 2000).

49 For test substances with adequate systemic availability (*i.e.* evidence for adequate availability  
50 to the target cells) there are several options for the *in vivo* testing:

- 1 • A rodent bone marrow or mouse peripheral blood micronucleus test (OECD TG 474) or  
2 a rodent bone marrow clastogenicity study (OECD TG 475). Potential species-specific  
3 effects may influence the choice of species and test method used.
- 4 • A transgenic rodent (TGR) mutation assay (OECD TG 488). TGR assays measure  
5 permanent gene mutations using reporter genes present in every tissue. In principle  
6 every organ can be sampled.
- 7 • A Comet (single cell gel electrophoresis) assay, which detects DNA strand breaks. This  
8 assay has the advantage of not being restricted to bone marrow cells. In principle every  
9 organ can be sampled. Although there is not yet an OECD guideline for this test,  
10 published guidance documents with respect to the design and performance of the test  
11 are available (Tice *et al.*, 2000; Hartmann *et al.*, 2003; McKelvey-Martin *et al.*, 1993;  
12 Brendler-Schwaab *et al.*, 2005; Burlinson *et al.*, 2007; Smith *et al.*, 2008; Rothfuss *et*  
13 *al.*, 2010; Burlinson, 2012; Vasquez, 2012; Johansson *et al.*, 2010; Kirkland & Speit,  
14 2008; EFSA, 2012). Other DNA strand breakage assays may be presented as  
15 alternatives to the Comet assay. All DNA strand break assays should be considered as  
16 surrogate tests, they do not necessarily detect permanent changes to DNA.
- 17 • A rat liver Unscheduled DNA synthesis (UDS) test (OECD TG 486). The UDS test is an  
18 indicator test measuring DNA repair. The UDS test should be considered as a surrogate  
19 test for an *in vivo* gene mutation test.

20 The *in vivo* genotoxicity test may be incorporated, if appropriate scientifically, into a short-  
21 term repeated dose toxicity test (28 days), for example, if this is to be performed to meet the  
22 requirements of the REACH Annex VIII tonnage level.

23 Any one of these tests may be conducted, but this has to be decided using expert judgement  
24 on a case-by-case basis. The nature of the original *in vitro* response(s) (*i.e.* gene mutation,  
25 structural or numerical chromosome aberration) should be considered when selecting the *in*  
26 *vivo* study. For example, if the test substance showed evidence of *in vitro* clastogenicity, then  
27 it would be most appropriate to follow this up with either a micronucleus test or chromosomal  
28 aberration test or a Comet assay. However, if a positive result were obtained in the *in vitro*  
29 micronucleus test, the rodent micronucleus test would be appropriate to best address  
30 clastogenic and aneugenic potential.

31 For chemicals that appear preferentially to induce gene mutations, the TGR assays are the  
32 most appropriate tests to follow-up an *in vitro* positive result and detect, *in vivo*, chemicals  
33 that induce gene mutation. The rat liver UDS test may also be suitable but its use should be  
34 justified on a case-by-case basis and take account of substance-specific considerations.  
35 Discussions on the recommended use the Comet assay are ongoing at the OECD level,  
36 however the applicability domain of the Comet assay seems to be similar to that of the TGR  
37 assays. The choice of any of these three assays can be justified only if it can be demonstrated  
38 that the tissue(s) studied in the assay is (are) exposed to the test substance (or its  
39 metabolites). This information can be derived from toxicokinetic data or, in case no  
40 toxicokinetic data are available, from the observation of treatment-related effects in the organ  
41 of interest. Another type of data that can support evidence of organ exposure is knowledge on  
42 the target organ(s) of specific classes of chemicals (*e.g.* the liver for aromatic amines). In case  
43 the *in vivo* Comet assay is used or proposed by the registrant, and since no adopted OECD TG  
44 is yet available, the test protocol followed or suggested should be described in detail and be in  
45 accordance with current scientific best practice, so as to ensure acceptability of the generated  
46 data. The TGR and Comet assays offer greater flexibility, most notably the possibility of  
47 selecting a range of tissues for study on the basis of what is known of the toxicokinetics and  
48 toxicodynamics of the substance. It should be realised that the UDS and Comet tests are  
49 indicator assays detecting putative DNA lesions. In contrast, the TGR gene mutation assays  
50 measure permanent mutations.

51 Additionally, evidence for *in vivo* DNA adduct formation in somatic cells together with *in vitro*  
52 test data may sometimes be sufficient to conclude that a substance is an *in vivo* somatic cell

1 mutagen. In such cases, positive *in vitro* test results may not trigger further *in vivo* somatic  
2 tissue testing, and the substance would be classified at least as a category 2 mutagen. The  
3 possibility for effects in germ cells would need further investigation (see Section [R.7.7.6.3](#),  
4 *Substances that give positive results in an in vivo test for genotoxic effects in somatic cells*).

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

9 For substances that are short-lived, reactive, *in vitro* mutagens, or for which no indications of  
10 systemic availability have been presented, an alternative strategy involving studies to focus on  
11 tissues at initial sites of contact with the body should be considered. Expert judgement should  
12 be used on a case-by-case basis to decide which tests are the most appropriate. The main  
13 options are the *in vivo* Comet assay, TGR gene mutation assays, and DNA adduct studies. For  
14 any given substance, expert judgement, based on all the available toxicological information,  
15 will indicate which of these tests are the most appropriate. The route of exposure should be  
16 selected that best allows assessment of the hazard posed to humans. For insoluble substances,  
17 the possibility of release of active molecules in the gastrointestinal tract may indicate that a  
18 test involving the oral route of administration is particularly appropriate.

19 At the time the update to this guidance was drafted (2013), a test guideline was still being  
20 prepared for one of the *in vivo* tests mentioned above, *i.e.* the Comet assay. In the absence of  
21 such a guideline, expert advice or appropriate alternative guidance, *e.g.* from the International  
22 Workshops on Genotoxicity Testing (IWGT, under the umbrella of the International Association  
23 of Environmental Mutagen Societies) should be sought about the conduct of these tests.

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

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

#### 34 [Substances that give positive results in an in vivo test for genotoxic effects in somatic cells](#)

35 Substances that have given positive results in cytogenetic tests *in vitro* and in such tests in  
36 somatic cells *in vivo* can be studied further to establish whether they specifically act as  
37 aneugens and thresholds for their genotoxic activity can be identified, if this has not been  
38 established adequately already. This should be done using *in vitro* methods and will be helpful  
39 in risk evaluation.

40 The potential for substances that give positive results in *in vivo* tests for genotoxic effects in  
41 somatic cells to affect germ cells should always be considered. The same is true for substances  
42 otherwise classified as category 2 mutagens. The first step is to make an appraisal of all the  
43 available toxicokinetic and toxicodynamic properties of the test substance. Expert judgement is  
44 needed at this stage to consider whether there is sufficient information to conclude that the  
45 substance poses a mutagenic hazard to germ cells. If this is the case, it can be concluded that  
46 the substance may cause heritable genetic damage and no further testing is justified.  
47 Consequently, the substance is classified as a category 1B mutagen. If the appraisal of  
48 mutagenic potential in germ cells is inconclusive, additional investigation will be necessary. In  
49 the event that additional information about the toxicokinetics of the substance would resolve  
50 the problem, toxicokinetic investigation (*i.e.* not a full toxicokinetic study) tailored to address  
51 this should be performed.

1 If germ cell testing is to be undertaken, and this should be in exceptional circumstances,  
2 expert judgement should be used to select the most appropriate test strategy. Internationally  
3 recognised guidelines are available for investigating clastogenicity in rodent spermatogonial  
4 cells and for the dominant lethal test. Dominant lethal mutations are believed to be primarily  
5 due to structural or numerical chromosome aberrations.

6 Alternatively, other methods can be used if deemed appropriate by expert judgement. These  
7 may include the Comet assay, TGR gene mutation assays, or DNA adduct analysis. In principle,  
8 it is the potential for effects that can be transmitted to the progeny that should be  
9 investigated, but tests used historically to investigate transmitted effects (the heritable  
10 translocation test and the specific locus test) use very large numbers of animals. They are  
11 rarely used and should not normally be conducted for industrial substances.

12 In order to minimise animal use, the possibility to combine germ cell genotoxicity tests and  
13 reproductive toxicity tests may be considered.

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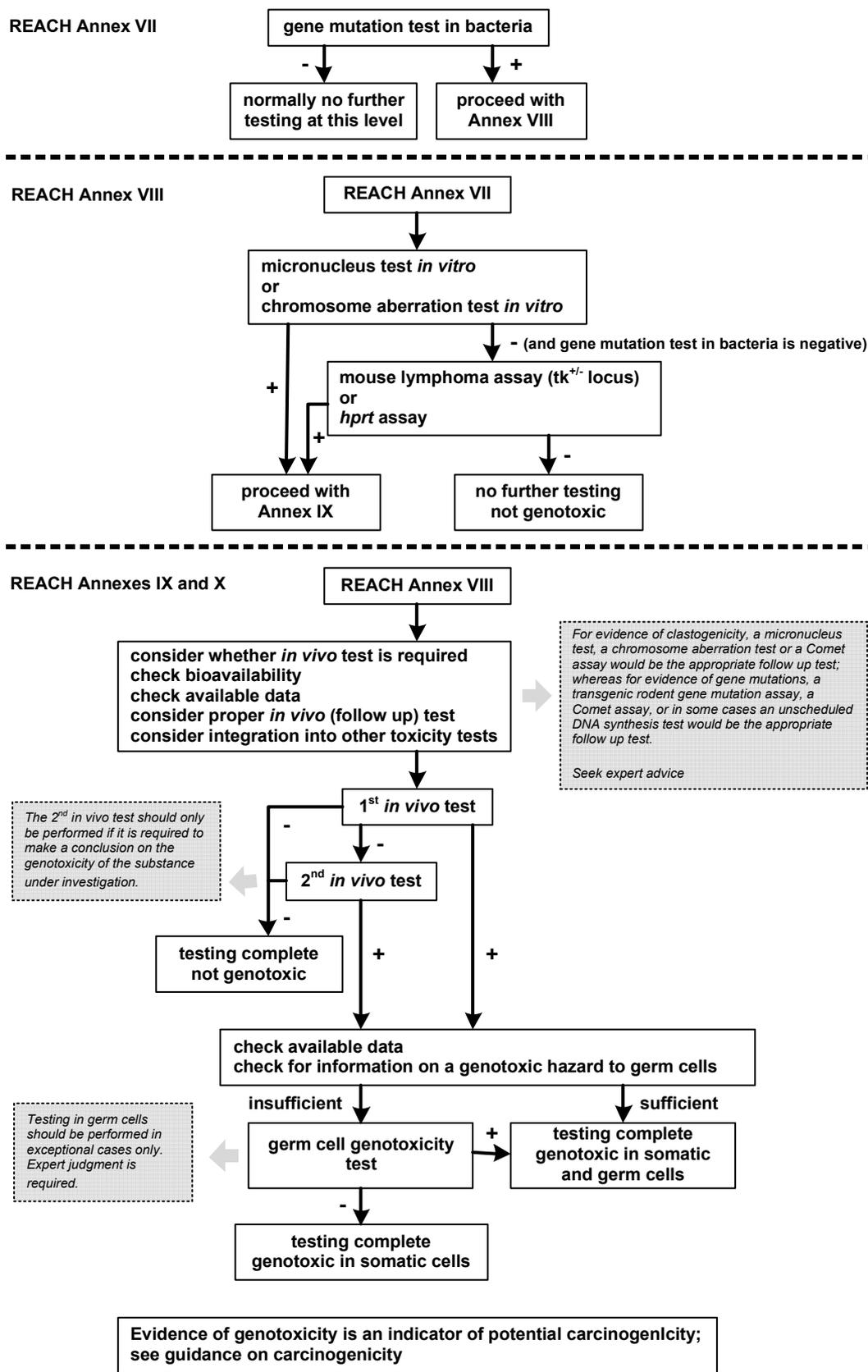
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1 **Figure R.7.7-1 Flow chart of the mutagenicity testing strategy**

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1 **Table R.7.7-5**

	<b>GM bact</b>	<b>Cytvitro</b>	<b>GMvitro</b>	<b>Cytvivo</b>	<b>GMvivo</b>	<b>Standard information required General follow up procedure</b>	<b>Conclusion</b>	<b>Specific rules for adaptation [for detailed guidance, incl. timing of the tests, see text]</b>	<b>Comments</b>
1	neg					Annex VII: no further tests are required. Annexes VIII, IX & X: conduct a CABvitro/MNTvitro, if this 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 effects on chromosome structure and number.
2	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 effects on chromosome structure and number.
3	neg		neg			Annex VII: no further tests are required. Annexes VIII, IX & X: conduct a CABvitro/MNTvitro	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 effects on chromosome structure and number.

	GM bact	Cytvitro	GMvitro	Cytvivo	GMvivo	Standard information required General follow up procedure	Conclusion	Specific rules for adaptation [for detailed guidance, incl. timing of the tests, see text]	Comments
4	neg	neg	neg			Annexes VII, VIII, IX & X: no further tests are required.	not genotoxic		The available metabolic evidence may, on rare occasions, indicate that <i>in vitro</i> testing is inadequate; <i>in vivo</i> testing is needed.  Seek expert advice.  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 effects on chromosome structure and number.
5	pos					Annexes VII, VIII, IX & X: Complete <i>in vitro</i> testing with a CABvitro/MNTvitro.			Consider need for further tests to understand the <i>in vivo</i> mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.

	GM bact	Cytvitro	GMvitro	Cytvivo	GMvivo	Standard information required General follow up procedure	Conclusion	Specific rules for adaptation [for detailed guidance, incl. timing of the tests, see text]	Comments
6	pos	neg				<p>Annexes VII &amp; VIII: <i>Complete in vitro</i> testing by conducting a GMvitro</p> <p>Annexes IX &amp; X: If systemic availability cannot be ascertained reliably, it should be investigated before progressing to <i>in vivo</i> tests.</p> <p>Select adequate somatic cell <i>in vivo</i> test to investigate gene mutations <i>in vivo</i> (TGR, or if justified UDSvivo or Comet)</p> <p>If necessary seek expert advice.</p>		Suspicion that a positive response observed in the GMbact was due to a specific bacterial metabolism of the test substance could be explored further by investigation <i>in vitro</i> .	<p>Ensure that all tests together with other available information enable thorough assessment for gene mutations and effects on chromosome structure and number.</p> <p>Consider on a case-by-case basis need for further tests to understand the <i>in vivo</i> mutagenicity hazard, to make a risk assessment, and to determine whether C&amp;L is justified.</p>
7	neg	pos				<p>Annexes VII, VIII, IX &amp; X: If systemic availability cannot be ascertained reliably, it should be investigated before progressing to <i>in vivo</i> tests.</p> <p>Select adequate somatic cell <i>in vivo</i> test to investigate structural or numerical chromosome aberrations (MNTvivo or Comet for <i>in vitro</i> clastogens and/or aneugens or CAbvivo for <i>in vitro</i>-clastogens)</p> <p>If necessary seek expert advice.</p>			<p>Ensure that all tests together with other available information enable thorough assessment for gene mutations and effects on chromosome structure and number.</p> <p>Consider need for further tests to understand the <i>in vivo</i> mutagenicity hazard, to make a risk assessment and to determine whether C&amp;L is justified.</p>

	GM bact	Cytvitro	GMvitro	Cytvivo	GMvivo	Standard information required General follow up procedure	Conclusion	Specific rules for adaptation [for detailed guidance, incl. timing of the tests, see text]	Comments
8	pos	pos				Annexes VII, VIII, IX & X: If systemic availability cannot be ascertained with acceptable reliability, it should be investigated before progressing to <i>in vivo</i> tests.  Select adequate somatic cell <i>in vivo</i> tests to investigate both structural or numerical chromosome aberrations and gene mutations.  If necessary seek expert advice.		Generally, both genotoxic endpoints should be investigated. If the first <i>in vivo</i> test is positive, a second <i>in vivo</i> test to confirm the other genotoxic endpoint need not be conducted.  If the first <i>in vivo</i> test is negative, a second <i>in vivo</i> test is required if the first test did not address the endpoints comprehensively.	Ensure that all tests together with other available information enable thorough assessment for gene mutations and effects on chromosome structure and number.  Consider need for further tests to understand the <i>in vivo</i> mutagenicity hazard, to make a risk assessment, and to determine whether C&L is justified.
9	pos neg	neg pos		neg	Neg	Annexes VII, VIII, IX & X: no further tests are required.	not genotoxic		Further <i>in vivo</i> test may be necessary pending on the quality and relevance of available data.
10	pos neg neg	neg pos neg		pos	Pos	Annexes VII, VIII, IX & X: No further testing in somatic cells is needed.  Germ cell mutagenicity tests should be considered.  If necessary seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the proper germ cell mutagenicity test.	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 this is the case, it can be concluded that the substance may cause heritable genetic damage and no further testing is justified.	If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation may be necessary.  Risk assessment and C&L can be completed.

11	pos	pos	(pos)	pos		Annexes VII, VIII, IX & X: No further testing in somatic cells is needed. Germ cell mutagenicity tests should be considered.	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 this is the case, it can be concluded that the substance may cause heritable genetic damage and no further testing is justified.	If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation may be necessary. Risk assessment and C&L can be completed.
	pos	pos	(pos)		Pos	If necessary seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the proper germ cell mutagenicity test.			
12	pos	pos	(pos)	neg		Annexes VII, VIII, IX & X: Select adequate somatic cell <i>in vivo</i> tests to investigate both structural or numerical chromosome aberrations and gene mutations. If necessary seek expert advice.			
	pos	pos	(pos)		Neg				
13	pos	pos	(pos)	neg	Neg	Annexes VII, VIII, IX & X: no further tests are required.	not genotoxic	Further <i>in vivo</i> test may be necessary pending on the quality and relevance of available data.	Risk assessment and C&L can be completed.
14	pos	pos	(pos)	neg	Pos	Annexes VII, VIII, IX & X: No further testing in somatic cells is needed. Germ cell mutagenicity tests should be considered.	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 this is the case, it can be concluded that the substance may cause heritable genetic damage and no further testing is justified.	If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation will be necessary. Risk assessment and C&L can be completed.
	pos	pos	(pos)	pos	Neg	If necessary seek expert advice on implications of all available data on toxicokinetics and toxicodynamics and on the choice of the proper germ cell mutagenicity test.			

- 1 Abbreviations: pos: positive; neg: negative; pos/neg: the follow up is independent from the result of
- 2 this test; GM<sub>bact</sub>: gene mutation test in bacteria (Ames test); Cyt<sub>vitro</sub>:cytogenetic assay in mammalian
- 3 cells; CAB<sub>vitro</sub>, *in vitro* chromosome aberration test; MNT<sub>vitro</sub>, *in vitro* micronucleus test; GM<sub>vitro</sub>:gene
- 4 mutation assay in mammalian cells; Cyt<sub>vivo</sub>:cytogenetic assay in experimental animals; GM<sub>vivo</sub>:gene
- 5 mutation assay in experimental animals;
- 6 CAB<sub>vivo</sub>, *in vivo* chromosome aberration test (bone marrow); MNT<sub>vivo</sub>, *in vivo* micronucleus test
- 7 (erythrocytes); UDS<sub>vivo</sub>, *in vivo* unscheduled DNA synthesis test; TGR, *in vivo* gene mutation test
- 8 with transgenic rodent; Comet, Comet assay.

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