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Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme

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Since the publication of the International Programme on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing, there have been a number of publications addressing test strategies for mutagenicity. Safety assessments of substances with regard to genotoxicity are generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation, clastogenicity and aneuploidy. It is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated that no single assay can detect all genotoxic substances. The World Health Organization therefore decided to update the IPCS Harmonized Scheme for Mutagenicity Testing as part of the IPCS project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals. The approach presented in this paper focuses on the identification of mutagens and genotoxic carcinogens. Selection of appropriate *in vitro* and *in vivo* tests as well as a strategy for germ cell testing are described.

Introduction

Since the publication of the International Programme on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing (1), there have been a number of publications addressing test strategies for mutagenicity (2–6) and reviews thereof (7). In addition, analyses of test batteries and their correlation with carcinogenicity (8–11) have indicated that an optimal solution to this issue has not yet been found. The 2005 International Workshop on Genotoxicity Testing (IWGT) meeting in San Francisco, USA, discussed many of these problems, and reports of this meeting (10,12) and companion papers (13–16) have recently been published.

Safety assessments of substances with regard to genotoxicity are generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation (i.e. point mutations or deletions/insertions that affect single or blocks of genes), clastogenicity (i.e. structural chromosome changes) and aneuploidy (i.e. numerical chromosome aberrations). It is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated that no single assay can detect all genotoxic substances. This is not surprising, as a wide variety of possible genetic events can occur. For example, some mutagens preferentially induce gene mutations by either base pair substitutions or frameshift mechanisms, whereas others induce chromosome mutations but show little or no evidence of inducing gene mutations.

The World Health Organization (WHO) therefore decided to update the IPCS Harmonized Scheme for Mutagenicity Testing (1) as part of the IPCS project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals. A public review draft paper was prepared by an International Drafting Group Meeting of experts, held at the Fraunhofer Institute for Toxicology and Experimental Medicine in Hanover, Germany, on April 11–12, 2007, and revised, following peer and public review, by an expert review meeting hosted by the University of Bradford, Bradford, UK, on June 30 to July 1, 2008. The present paper is the product of the expert review meeting.

Strategy for mutagenicity testing

The approach presented in this paper (see Figure 1) focuses on the identification of mutagens and genotoxic carcinogens. The term ‘mutation’ as understood in this paper (a glossary of terms used in this paper is available on the IPCS website at http://www.who.int/ipcs/publications/methods/harmonization/en/index.html) refers to permanent changes in the structure and/or amount of the genetic material of an organism that can lead to heritable changes in its function, and it includes gene mutations as well as structural and numerical chromosome alterations. The group is aware of other mechanisms leading to carcinogenicity and other heritable diseases, but their identification requires additional types of mechanistic studies. ‘Genotoxicity’ refers to the capability of substances to damage DNA and/or cellular components regulating the fidelity of the genome—such as the spindle apparatus, topoisomerases, DNA repair systems and DNA polymerases (4)—and includes all adverse effects on genetic information. These potentially harmful effects on genetic material may be mediated directly or

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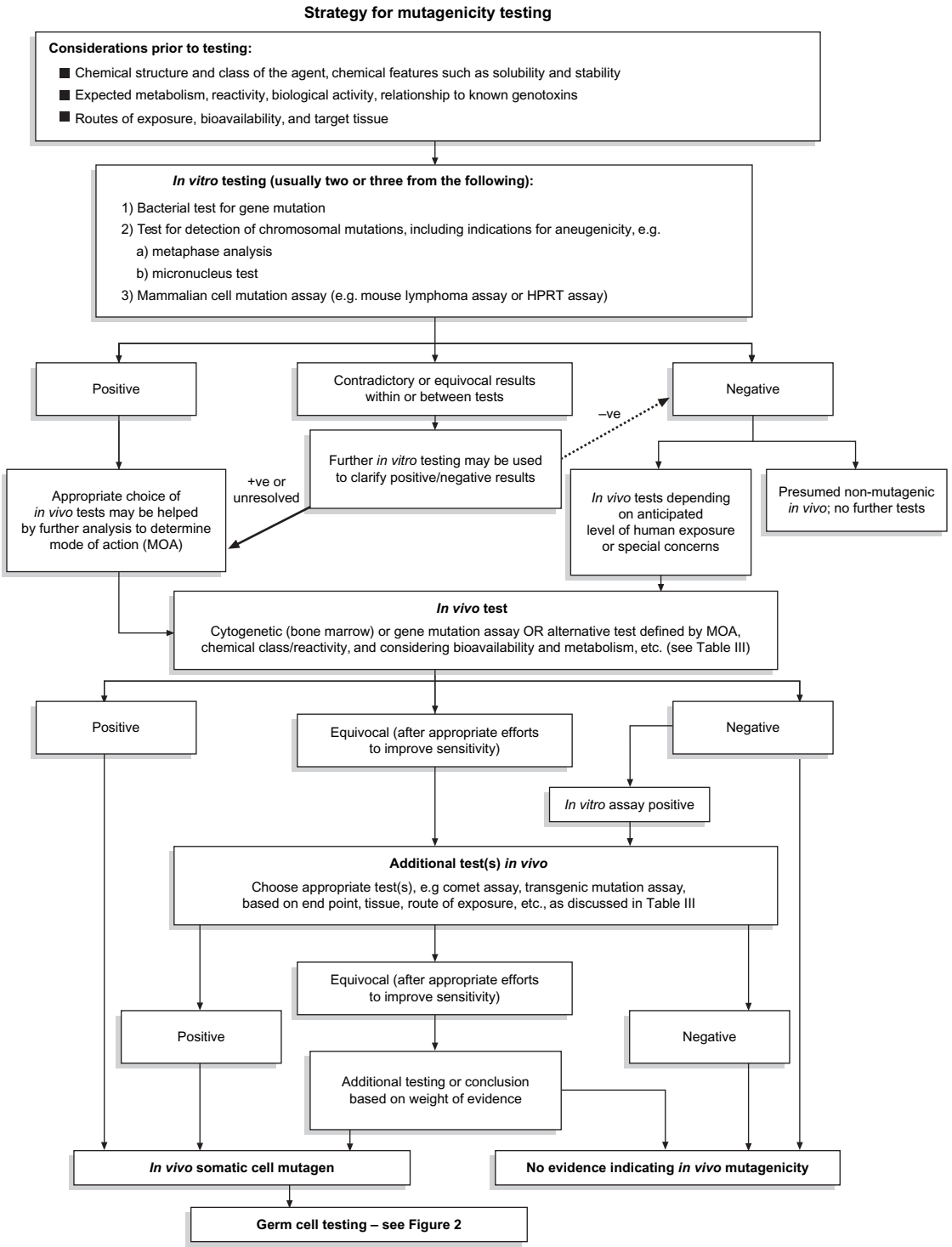

Fig. 1. Strategy for mutagenicity testing.

indirectly and are not necessarily associated with mutagenicity. Genotoxicity is therefore a broader term than 'mutagenicity', which refers to the capacity to give rise to mutations.

Because of the wide range of genetic damage that can occur, test batteries are designed to include complementary tests evaluating different mechanisms of mutagenicity. At all stages of the outlined testing strategy, a weight of evidence approach and scientific judgement should be used. Multiple negative results may not be sufficient to remove concern for mutagenicity raised by a clear positive result in a single mutagenicity assay.

Most short-term tests in bacteria and mammalian cell cultures have been designed primarily for hazard identification and thus can represent only the starting point in the process of risk assessment. Whether or not the observed effects are relevant for humans under anticipated exposure conditions depends on pharmacokinetic, pharmacodynamic and other factors that require investigation *in vivo*.

Especially when choosing *in vivo* assays and when proceeding into germ cell mutagenicity studies (see Strategy for germ cell testing), expert judgement is required to select the

appropriate test systems and to avoid uninformative and thus unnecessary animal experiments.

Development of a testing strategy

Before initiating mutagenicity testing on a particular substance (or mixture of substances), the following aspects should be considered, when available:

(i) Chemical structure and class of the substance (possible structure–activity relationships) and physicochemical properties, such as solubility and stability;
(ii) Expected pathways of metabolism, chemical and biological reactivity/activity and relationship to known genotoxic substances and
(iii) Routes of exposure, bioavailability and target tissues for genotoxicity.

Critical evaluation of available data prior to testing usually provides important information for choosing the appropriate *in vitro* assays, but even more so for the selection of appropriate *in vivo* studies.

Distinction needs to be made between 'mutagenicity tests' in the strict sense and 'indicator tests' that provide evidence of interaction with DNA that may or may not lead to mutations (e.g. DNA adducts, DNA strand breaks and sister chromatid exchanges). Preference should be given to mutagenicity tests whenever possible.

In vitro testing

Usually two or three different tests in bacteria and mammalian cells are selected to cover the end points of gene mutations, clastogenicity (structural chromosome aberrations) and aneuploidy (numerical chromosome aberrations), taking into account physicochemical properties of substances under consideration.

In vitro tests. Screening should be based on a limited number of tests that are well validated and informative. Genotoxicity test batteries generally include the following:

(i) A test for gene mutation in bacteria (bacterial reverse mutation assay): Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 recommends the use of at least five strains of bacteria: (a) *Salmonella typhimurium* TA1535, (b) *S.typhimurium* TA1537 or TA97 or TA97a, (c) *S.typhimurium* TA98, (d) *S.typhimurium* TA100 and (e) *Escherichia coli* WP2 or *E.coli* WP2*uvrA* or *S.typhimurium* TA102. The choice of additional tests depends on the chemical structure and class of the substance (see Development of a testing strategy). Table I describes the most commonly used bacterial mutagenicity tests.
(ii) *In vitro* mammalian assays: These assays should evaluate the potential of a substance to induce point mutations, clastogenicity and/or aneugenicity, by using either mammalian cell lines or primary human cell cultures such as fibroblasts or lymphocytes (e.g. mouse lymphoma thymidine kinase assay, hypoxanthine guanine phosphoribosyltransferase assay or cytogenetic evaluation of chromosomal damage in mammalian cells via either the *in vitro* chromosome aberration or the *in vitro* micronucleus test) (see Table II).

Evaluation of in vitro testing results. In the evaluation, results are classified into (i) positive, (ii) negative and (iii) contradictory or equivocal:

(i) Positive: Substance is positive at one or more end points of mutagenicity.
(ii) Negative: Substance is negative in all test systems under appropriate *in vitro* test conditions; the substance is not mutagenic (or genotoxic) *in vitro* and is anticipated not to be mutagenic *in vivo* [for exceptions, see refs (37,38)].
(iii) Contradictory or equivocal (e.g. borderline biological or statistical significance): All other substances.

Follow-up to in vitro testing.

(i) Positive *in vitro* results
In vivo test; selection of an appropriate end point; if necessary, further *in vitro* studies to optimize *in vivo* testing (e.g. kinetochore staining as an addition in the micronucleus assay of *in vitro* aneugens). Follow-up tests *in vitro* may also provide additional mechanistic information to enable interpretation of a positive finding.
(ii) Negative *in vitro* results
In vivo testing is recommended in the case of 'high' or 'moderate and sustained' human exposure or for substances otherwise of high concern. In limited cases, metabolic considerations may trigger *in vivo* testing (38).
(iii) Contradictory or equivocal *in vitro* results
Further *in vitro* testing to clarify positive or negative results; depending on whether the situation is resolved by further *in vitro* testing, proceed according to 'positive' or 'negative'.

In vivo testing

In vivo tests. In vivo tests (see Tables III and IV) should be chosen carefully to avoid an uninformative outcome and with concern for animal welfare. Therefore, toxicokinetics, metabolism and chemical reactivity have to be considered carefully. *In vivo* tests may also be used for evaluation of a dose–response, species differences or mode of action determination. The use of such tests needs to be considered on a case-by-case basis for risk assessment purposes.

The choice of an *in vivo* follow-up test should be guided by the spectrum of genotoxic events observed in the *in vitro* studies as well as knowledge of the bioavailability, distribution, metabolism and target organ specificity of the substance. Typically, a bone marrow micronucleus or clastogenicity test is conducted. However, if there are indications that point to a more appropriate assay, then this assay should be conducted instead (e.g. mutagenicity study with transgenic animals and/or comet assay in potential target tissues).

Follow-up to in vivo testing.

(i) Positive *in vivo* results
Substance is considered an '*in vivo* somatic cell mutagen'. Testing for germ cell mutagenicity (see Strategy for germ cell testing) may be required.

Table I. Common *in vitro* bacterial assays

Assay	Strain	End point	Comments	Published guidelines	References
Salmonella typhimurium reverse mutation assay	TA1535, TA1537 (or TA97 or TA97a), TA98, TA100	Primarily detects G/C base pair and frameshift mutations	Contain specific mutations in one of several genes involved in histidine biosynthesis that must be reverted to function normally. Testing with and without appropriate exogenous metabolic activation system. May not detect some oxidizing mutagens and cross-linking agents.	OECD Test Guideline 471 (replaces old OECD Test Guidelines 471 and 472)	(17–19)
S.typhimurium	TA102	Primarily detects A/T base pair damage and small deletions	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19,20)
Other *S.typhimurium* mutants	YG1021, YG1026 (NR overexpression); YG1024, YG1029 (NAT overexpression)		For detection of mutagenicity of nitroaromatic and aminoaromatic substances that are bioactivated by NR and NAT. More sensitive than conventional strains. Used for detecting mutagenicity of toxic pollutants in air, water and food.		(21,22)
Escherichia coli reverse mutation assay	WP2, WP2*uvrA*	Primarily detects A/T base pair damage	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19)

A, adenine; C, cytosine; G, guanine; NAT, *N*-acetyltransferase; NR, nitroreductase; T, thymine.

Table II. Common *in vitro* mammalian assays

Assay	Method/end point	Main attributes	Comments	Published guidelines	References
Mouse lymphoma TK gene mutation assay	L5178Y mouse lymphoma cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also various sizes of chromosome deletions and other effects that can lead to loss of heterozygosity (e.g. mitotic recombination, gene conversion and translocations)	Use of positive controls and colony sizing essential for quality control. Evaluation and interpretation changed over the years. Recent protocol updates recommendations. Can be used as alternative to metaphase analysis.	OECD Test Guideline 476; IWGT guidelines	(3,23–26)
HPRT gene mutation assay	Chinese hamster ovary, AS52 or other suitable cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also small deletions; larger deletions may be detected in AS52 cells	Use of positive controls essential for quality control	OECD Test Guideline 476	(23,27)
Metaphase analysis (*in vitro* mammalian chromosome aberration test)	A metaphase-arresting substance (e.g. colchicine) is applied; metaphase cells are analysed for the presence of structural chromosome aberrations	Detects clastogenicity; some information on aneugenicity can be obtained with extended culture times	A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes) (28)	OECD Test Guideline 473	(29–31)
Micronucleus test	Detects micronuclei in the cytoplasm of cultured mammalian cells during interphase	Detects both aneugenic and clastogenic substances; established mammalian lines, cultured human peripheral blood lymphocytes or Syrian hamster embryo cells may be used	Several developments in updating the protocol. Immunochemical labelling of kinetochores or hybridization with general or chromosome-specific centromeric/telomeric probes gives information on the nature and mechanism of formation of micronuclei induced (whole chromosomes or fragments).	Draft OECD Test Guideline 487	(13,32–36)

HPRT, hypoxanthine guanine phosphoribosyltransferase; TK, thymidine kinase.

Table III. Common *in vivo* genotoxicity assays

Assay	End point	Main attributes	Comments	Published guidelines	References
Micronucleus test in erythropoietic cells	Structural and numerical chromosome alterations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 474	(15,28), and references cited therein
Metaphase analysis *in vivo*	Structural and numerical chromosome aberrations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 475	(39)
Transgenic animal models	Gene mutation	Can be applied to many tissues. Gene specific. No selective pressure on mutations. Relevant end point.	Need to optimize protocols overall and for each tissue. *lacI*, *lacZ*, *gpt* systems not sensitive to the detection of large deletions. Spi^- system detects large deletions.	IWGT, IPCS guidance	(40–44)
Chemically modified DNA	Covalent DNA adducts, oxidative lesions (e.g. 8-OH-dG)	Can be applied to many tissues. Can be highly sensitive (^{32}P-postlabelling or AMS) or chemically specific (MS). Other methods include immunochemical techniques, fluorescence, ECD (for 8-OH-dG).	Indicator test detecting premutagenic lesions. Interpretation of results can be complicated.	IWGT guidance	(45)
DNA strand breakage assays (e.g. comet assay)	DNA strand breaks, alkali-labile lesions	Can be applied to many tissues. Incorporation of enzymes can improve specificity. Cell division not required.	Indicator tests. Need to optimize protocols for different tissues. May be unable to detect mutagens that do not induce strand breaks or alkali-labile lesions, but may detect repair-induced breaks. Apoptosis/necrosis need to be controlled.	IWGT guidance	(14,46–49)
Liver UDS	Thymidine incorporation outside S phase	Long history of use; useful for some classes of substances.	Indicator test detecting repair activity. Uncertain acceptability and questionable sensitivity. Limited use in other tissues.	OECD Test Guideline 486	(50,51)

8-OH-dG, 8-hydroxy-2′-deoxyguanosine; AMS, accelerator mass spectrometry; ECD, electrochemical detection; MS, mass spectrometry; UDS, unscheduled DNA synthesis.

(ii) Negative *in vivo* results
Further *in vivo* testing is recommended in the case of positive *in vitro* studies. Again, the second *in vivo* test is chosen on a case-by-case basis, as stated above. If the test is negative, it is concluded that there is no evidence for *in vivo* mutagenicity.

(iii) Equivocal *in vivo* results
Equivocal results may be due to low statistical power, which can be improved by increasing the number of treated animals and/or scored cells.
If the situation is unresolved, a second *in vivo* test is recommended, chosen on a case-by-case basis (ordinarily on a different end point or in a different tissue, depending on toxicokinetics, metabolism and mode of action); proceed according to 'positive' or 'negative'.

Strategy for germ cell testing

When information on the risk to the offspring of exposed individuals is important, the following germ cell testing strategy is recommended.

For substances that give positive results for mutagenic effects in somatic cells *in vivo*, their potential to affect germ cells should be considered. If there is toxicokinetic or toxicodynamic evidence that germ cells are actually exposed to the somatic mutagen or its bioactive metabolites, it is reasonable to assume that the substance may also pose a mutagenic hazard to germ cells and thus a risk to future generations.

Where germ cell testing is indicated, judgement should be used to select the most appropriate test strategy. There are a number of tests available (summarized in Table IV), which fall into two classes:

(i) Tests in germ cells *per se* (class 1)
(ii) Tests to detect effects in the offspring (or potential offspring) of exposed animals (class 2)

Three tests that are available for such studies have established OECD test guidelines:

(i) Clastogenicity in rodent spermatogonial cells (class 1): OECD Test Guideline 483 (65)

Table IV. Germ cell assays

Assay	End point	Main attributes[a]	Comments	Published guidelines	References
Class 1: tests in germ cells *per se*					
Transgenic animal models	Gene mutation	Gene specific. No selective pressure on mutations. Relevant end point.	See Table III	See Table III	See Table III
ESTR assay	Non-coding tandem repeat DNA mutation	Potentially relevant end point. Detects heritable mutations at ambient exposure levels. Uses relatively few animals. Can be conducted in humans.	Some tandem repeat mutations also occur in, or near, coding genes. Although there are parallels with mutations in coding genes, the human health outcomes require further study.		(52–55)
Mammalian spermatogonial chromosome aberration test	Structural chromosome aberrations	Relevant end point		OECD Test Guideline 483	(56)
FISH assays	Structural chromosome aberrations; sperm aneuploidy	Relevant end points. Can be conducted in humans.	See Table III	See Table III	(57,58)
Comet assay	DNA strand breaks or alkali-labile sites	See Table III. Can be conducted in humans.	See Table III	See Table III	(59)
Chemically modified DNA	DNA adducts	See Table III. Can be conducted in humans.	See Table III	See Table III	(60)
Class 2: tests to detect effects in the offspring (or potential offspring)					
ESTR assay	As above for class 1 tests	As above for class 1 tests	As above for class 1 tests		As above for class 1 tests
Dominant lethal test	Reduction in viable embryos attributed to chromosome or gene mutations	Relevant end point. Provides data for quantification of pregnancy loss.		OECD Test Guideline 478	(61)
Mouse visible specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5200	(62)
Mouse biochemical specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5195	(63)
Mouse heritable translocation assay	Structural chromosome aberrations	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	OECD Test Guideline 485	(64)

EPA OPPTS, United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances; ESTR, Expanded Simple Tandem Repeat; FISH, fluorescence *in situ* hybridization.
[a]'Relevant end point' means relevant to the estimation of human heritable health risk.

(ii) The dominant lethal test (class 2): OECD Test Guideline 478 (66)
(iii) The mouse heritable translocation assay (class 2): OECD Test Guideline 485 (67)

The above-mentioned class 2 tests usually require large numbers of animals. Thus, in order to minimize the use of animals in germ cell testing, it is advisable to start with tests that detect effects in germ cells *per se* (class 1). Other methods include (but are not limited to) gene mutation tests in transgenic animals [see ref. (41) for IWGT guidance], gene mutations in the more recent Expanded Simple Tandem Repeat (ESTR) assay, chromosomal assays (including those using fluorescence *in situ* hybridization), comet assay and DNA adduct analysis.

Following the use of such tests, if quantification of heritable effects is required (class 2), an assay for ESTR mutations can be performed with the offspring of a low number of exposed animals. Tests used historically to investigate transmitted effects (e.g. the heritable translocation test and the specific locus test) can also be performed; however, they use large numbers of animals.

Class 1 and class 2 germ cell assays are summarized in Table IV. The strategy used in germ cell mutagenicity testing is outlined in Figure 2.

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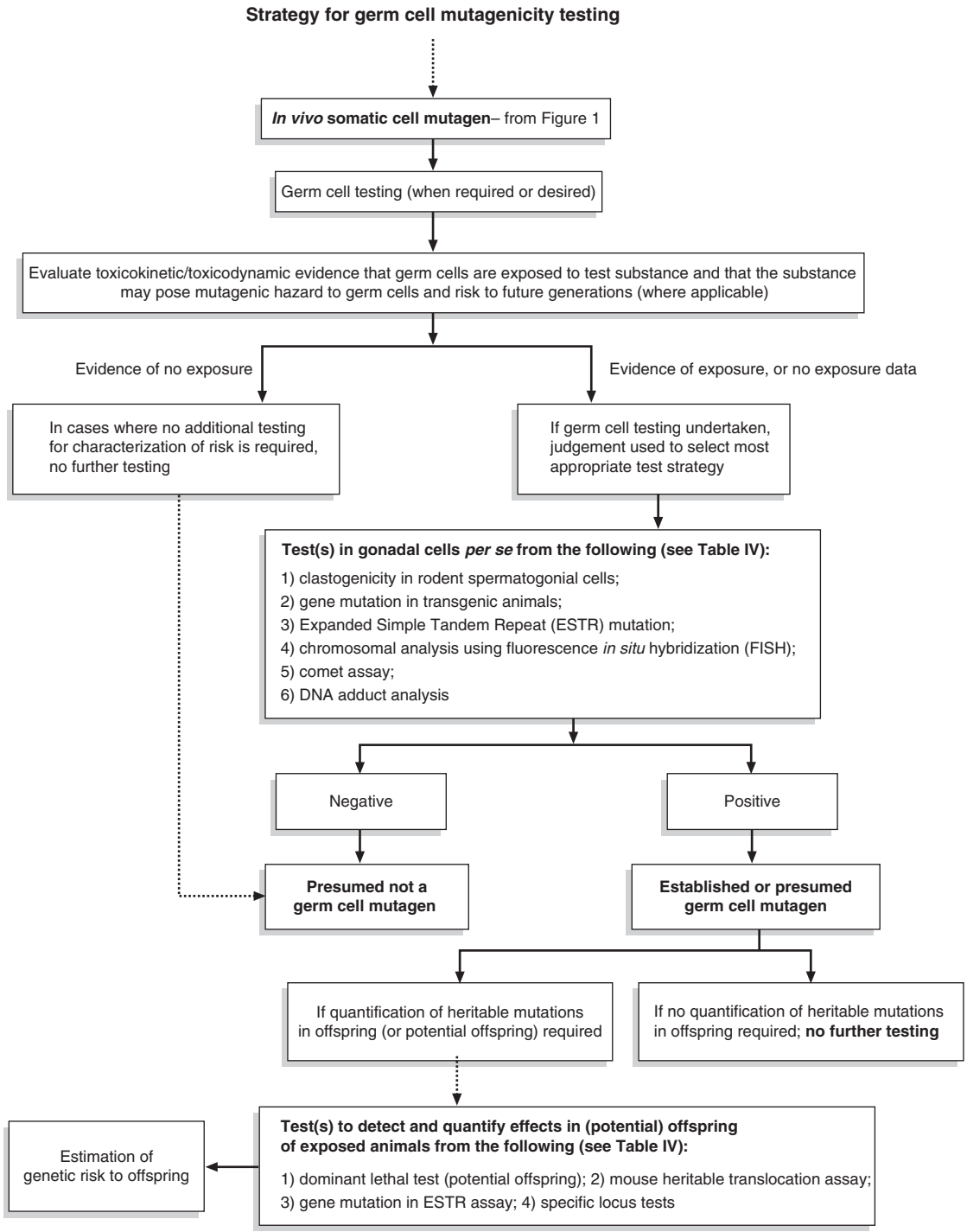

Fig. 2. Strategy in germ cell mutagenicity testing.

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