

This is a draft paper for consultation. All comments should be sent to [Sue.Kennedy@hpa.org.uk](mailto:Sue.Kennedy@hpa.org.uk) as early as possible and by 14 February 2011 at the latest.

## GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING AND MUTAGENIC HAZARD ASSESSMENT OF CHEMICAL SUBSTANCES

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3 **GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING**  
4 **AND MUTAGENIC HAZARD ASSESSMENT OF CHEMICAL**  
5 **SUBSTANCES**

6 **I. Preface**

7 1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products  
8 and the Environment (COM) is an expert advisory committee whose members  
9 are appointed by the Chief Medical Officer for England and the Chair of the  
10 Food Standards Agency (FSA) following an appointments exercise involving  
11 public advertisement. Members serve in their own capacity as independent  
12 experts and observe a published code of practice including principles relating  
13 to the declaration of possible conflicting interests.

14

15 2. The remit of the COM is to advise all U.K. government departments and  
16 agencies with an interest in the safety of chemicals across various sectors, on  
17 the human health aspects of the mutagenicity and genotoxicity of chemicals.  
18 (These terms are defined for the purposes of this guidance document in  
19 paragraphs 7-8 below.) The Secretariat is provided by the Health Protection  
20 Agency (HPA) (who lead) and the Food Standards Agency. Other  
21 government departments with an interest provide assessors to the COM; these  
22 are specifically from the Department of Health (DH) the Department of  
23 Environment, Food and Rural Affairs (Defra), the Chemicals Regulation  
24 Directorate (CRD) of the Health and Safety Executive (HSE) (responsible for  
25 legislation regulating chemicals, pesticides, biocides and detergents), the  
26 Environment Agency, the Veterinary Medicines Directorate (VMD: a Defra  
27 agency responsible for the licensing of veterinary drugs) and the Medicines  
28 and Healthcare products Regulatory Agency (MHRA; a DH agency  
29 responsible for the licensing of human medicines). In addition there are  
30 assessors from the Scottish Government, the Welsh Assembly Government  
31 and the Northern Ireland Assembly.

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3. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to an agency that does have such a role (e.g. HSE CRD for occupational aspects and for pesticides etc). Its remit is to advise on the human health aspects of mutagenicity and genotoxicity of chemicals, and this may involve advice on a specific chemical, and also on testing strategies and research. Throughout this guidance the COM has referred to the genotoxicity testing of substance(s). In this document the term substance refers to a specified chemical including any additive necessary to preserve its stability and any impurity deriving from the process used. (<http://www.hse.gov.uk/reach/definitions.htm#substance>). However the COM usually provides advice on a specific chemical substance which can be equated to a single chemical or compound or pure substance. (<http://www.iupac.org/objID/Source/sou17657978492176660060882>). The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with mutagenic and genotoxic hazards (inherent properties of chemicals) or risk (the likelihood of mutagenic or genotoxic effects occurring after a given exposure to a chemical) and to present recommendations for genotoxicity testing. In practice the bulk of the work of the COM relates to assessing genotoxicity tests and providing advice on mutagenic hazard of chemicals.
  
4. In the context of testing strategies the COM first published guidelines for the testing of chemicals for mutagenicity in 1981 and these were revised in 1989 (DOH., 1989). These provided guidance to the relevant government departments and agencies on best practice for testing at that these times. The need for guidance to be periodically updated, to reflect advances in development and validation of methods, was recognised and revised guidance was published in 2000 (DOH., 2000). This new guidance continues this updating process. The strategy outlined in this guidance is considered to be the most scientifically appropriate given available methods and recognises the need to avoid use of live animals where practical and where validated alternative methods are available. It is recognised that, as with the earlier

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1 published COM guidance, it may be some time before this strategy is reflected  
2 in guidelines used by UK regulatory authorities. Specific guidance is now also  
3 given for applying the COM testing strategy to the mutagenicity assessment of  
4 chemical substances which have existing, but in many cases inadequate or  
5 incomplete genotoxicity data.

6 5. The COM believes that the approach outlined presents an overview of the core  
7 principles of genotoxicity testing and will remain valid for several years. It is  
8 acknowledged that existing national or international testing strategies will be  
9 at different stages of review and hence inconsistencies are expected. The  
10 COM guidance is not intended to supersede or replace existing national or  
11 internationally sector specific genotoxicity testing strategies (e.g. those  
12 recommended for pharmaceuticals by the International Conference on  
13 Harmonisation of Technical Requirements for Registration of Pharmaceuticals  
14 for Human Use (ICH) (<http://www.ich.org/cache/compo/276-254-1.html>) and  
15 for chemicals assessed under the Registration, Evaluation, Authorisation and  
16 Restriction of Chemicals (REACH) Regulation (EC1906/2006)  
17 ([http://guidance.echa.europa.eu/docs/guidance\\_document/information\\_requirements\\_en.htm](http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm)).  
18

## 19 **II. Introduction**

20 6. The COM last published guidance on a strategy for the testing of chemicals for  
21 mutagenic potential in 2000 (DOH., 2000). The rationale developed by COM  
22 in 2000, particularly in relation to the testing of all potential mutagenic  
23 endpoints, has also been adopted by the International Workshops on  
24 Genotoxicity Testing (IWGT) (Müller et al., 2003b). Since 2000 there has  
25 been development of new approaches to identifying genotoxic hazards *in vitro*  
26 including new approaches to identify misleading positive results and evaluate  
27 target organ genotoxicity *in vivo*. There is also a need to develop a testing  
28 strategy which can encompass chemicals such as cosmetics where no animal  
29 tests are permitted under EU law. It is the objective of this paper to set out a  
30 scientifically valid testing strategy comprising those methods which are  
31 believed to be the most informative and (when possible) are well validated.  
32 There is no discussion of methods which experience has shown to have no

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1 place in the recommended genotoxicity testing strategy. Details of  
2 methodologies are not given since they are provided in the Organisation for  
3 Economic Cooperation and Development (OECD) test guidelines), the EU  
4 Test Methods Regulation (EC 440/2008) [http://eur-](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:142:0001:0739:en:PDF)  
5 [lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:142:0001:0739:en:P](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:142:0001:0739:en:PDF)  
6 [DF](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:142:0001:0739:en:PDF) and the International Workshops on Genotoxicity Testing (IWGT)  
7 guidance.

8 7. The COM evaluates the results of the available tests on a particular substance  
9 and provides advice on mutagenic hazard (i.e. mutagenic potential). The term  
10 “mutagenic” refers to the ability of a substance to induce a permanent change  
11 in the amount or structure of the genetic material of an organism, which may  
12 result in a heritable change in the characteristics of the organism. Chemicals  
13 inducing mutations are referred to as mutagens (they are mutagenic). These  
14 alterations may involve individual genes, blocks of genes, or whole  
15 chromosomes. Mutations involving single genes may be a consequence of  
16 effects on single DNA bases (point mutations) or of larger changes, including  
17 deletions and rearrangements of DNA. The potential to induce mutation is  
18 measured in test systems that detect a broader range of genetic changes than  
19 simply mutation – they measure genotoxicity.

20 8. Genotoxic (or genotoxicity) refers to chemicals that interact with or damage  
21 the DNA and/or the cellular apparatus which regulates the fidelity of the  
22 genome, e.g. the spindle apparatus, and enzymes such as the topoisomerases.  
23 It is a broad term that, as well as mutation, includes structural chromosomal  
24 damage (clastogenicity), numerical chromosomal damage (aneuploidy,  
25 polyploidy) damage to DNA or the production of DNA adducts, by the  
26 chemical itself or its metabolites. Genotoxic effects also include DNA strand  
27 breakage, unscheduled DNA synthesis (UDS), sister chromatid exchange  
28 (SCE) and mitotic recombination in yeast. However the detection of such  
29 effects does not in itself provide direct evidence of inherited mutations. The  
30 term “genotoxic carcinogen” as used by the COM described those chemicals  
31 that have been demonstrated to be carcinogenic in humans and/or animals and

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1 are considered to be *in vivo* mutagens. Mutagenicity is accepted as a key  
2 event in carcinogenicity.

3 9. The objective of genotoxicity testing is to exclude or identify potential  
4 mutagenic hazards to humans, and, for those that are positive, to aid in the  
5 elucidation of the mode of genotoxic action (MoGA). This guidance therefore  
6 presents a strategy for genotoxicity testing since this term encompasses all the  
7 assays included in the strategy. Consequently, it is important to generate  
8 information on three levels of genetic damage, namely gene mutation,  
9 chromosome structure (i.e. clastogenicity) and chromosome number (i.e.  
10 aneuploidy), to provide comprehensive coverage of the mutagenic potential of  
11 a chemical.

12 10. The COM reaffirms its view published in 1989 and 2000, that there is  
13 currently no single validated assay that can provide comprehensive  
14 information on all three levels of genetic damage and thus it is necessary to  
15 subject a given substance to several different assays. A range of assays have  
16 been developed which employs a wide variety of organisms, including  
17 prokaryotes (bacteria), yeasts and other eukaryotic microorganisms, and  
18 mammalian cells studied *in vitro*, as well as whole mammals where effects in  
19 a wide range of target organs including germ cells can be measured. Assays  
20 may be classified on the basis of genetic end-points (e.g. gene mutation,  
21 clastogenicity, aneugenicity and tests for DNA damage) or by consideration of  
22 the different phylogenetic levels (e.g. bacteria, and mammalian cell)  
23 represented and also in mammals by the tissues or target organs studied.  
24

### 25 **III Significance of Chemical Induced Mutation for Human Health**

26 11. A mutation in the germ cells of sexually reproducing organisms may be  
27 transmitted to the offspring, whereas a mutation that occurs in somatic cells  
28 may be transferred only to descendent daughter cells. Mutagenic chemicals  
29 may present a hazard to health since exposure to a mutagen carries the risk of  
30 inducing germ-line mutations, with the possibility of inherited disorders, and  
31 the risk of somatic mutations including those leading to cancer.

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- 1 12. A separate guidance statement on the significance of chemical induced  
2 mutation to human health is in preparation.

3 **IV. General Principles of Testing Strategy**

- 4 13. The COM recommends a two-stage genotoxicity testing strategy (Stages 1 and  
5 2) for the detection of mutagenic hazard of chemicals which can be supported  
6 by appropriate preliminary screening tests and/or *in silico* data (Stage 0).  
7 Initial testing for mutagenic potential in Stage 1 is based upon two core *in*  
8 *vitro* tests that are chosen to provide information on gene mutation,  
9 clastogenicity and aneuploidy, with case-by-case additional testing and  
10 investigation depending on the results of these initial genotoxicity tests. All *in*  
11 *vitro* tests should be designed to provide the best chance of detecting potential  
12 activity, with respect to (a) the exogenous metabolic activation system (S9 -  
13 see glossary); (b) the ability of the compound or its metabolite(s) to reach the  
14 target DNA and/or targets such as the cell division apparatus, and (c) the  
15 ability of the genetic test system to detect the given type of genotoxic event.  
16 Where international guidance is available, the assays should be carried out to  
17 conform to these internationally recognised protocols (e.g. as published by the  
18 OECD, the IWGT and in the EU test methods Regulation (EC 440/2008). The  
19 same approach to testing can be used for chemical substances where *in vivo*  
20 genotoxicity testing is not permitted (e.g. cosmetics). A case-by-case testing  
21 strategy should be developed for substances which have existing but possibly  
22 inadequate and/or incomplete genotoxicity data. Investigations regarding  
23 mode of genotoxic action are important to derive conclusions on biological  
24 significance of genotoxicity tests and to inform on the strategy for *in vivo* tests  
25 and are particularly important for those chemicals where no *in vivo*  
26 genotoxicity testing is permitted.
- 27 14. For most chemicals negative results from the two Stage 1 core tests should  
28 be sufficient to reach conclusion on the presence or absence of mutagenic  
29 potential. However, in some instances regulatory authorities may require  
30 consideration of the need for *in vivo* Stage 2 testing where exposure is  
31 considered to be high, or moderate and prolonged (e.g. most human  
32 medicines). Guidance on the level of exposure which equates to high,

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1 moderate or prolonged is beyond the scope of COM guidance. However, if  
2 exposures exceed the Threshold of Toxicological Concern (TTC), which has  
3 been used as a risk assessment tool predominantly in the food and  
4 pharmaceutical sectors, this may be useful to identify priorities for further  
5 testing on a case-by-case basis (Munro et al., 2008).

6 15. Stage 2 consists of a number of *in vivo* tests designed to investigate whether  
7 *in vitro* genotoxic activity including specific mutagenic end points identified  
8 by *in vitro* tests can be expressed in the whole animal. This may also  
9 include assays for specific target organs (e.g. rodent tumour organs) or in  
10 germ cells. There is currently no single *in vivo* test which can assay all three  
11 levels of genetic damage (Thybaud et al., 2007) and thus a strategy for  
12 Stage 2 has to be designed based on the nature of genotoxic effects identified  
13 in Stage 1.

14 16. There should be a clear strategy for planning tests within each stage and for  
15 progressing to Stage 2. Clear statements can be made regarding the initial *in*  
16 *vitro* tests to be used in Stage 1 as these methods have been well studied  
17 whereas the strategy for Stage 2 is more complex and needs to be developed  
18 on a case-by-case basis.

19 17. Few chemicals are active only *in vivo* and in such cases this may be due to a  
20 number of factors such as metabolic differences, the influence of gut flora,  
21 higher exposures *in vivo* compared to *in vitro* and pharmacological effects  
22 (e.g. folate depletion or receptor kinase inhibition) (Tweats et al., 2007b).

23 18. Under the strategy recommended by COM, the use of animals in mutagenicity  
24 testing is primarily required when it is necessary to investigate whether  
25 genotoxic activity detected in Stage 1 *in vitro* is reproduced *in vivo*, to study  
26 target organ genotoxicity (for example involvement of genotoxicity in rodent  
27 tumours(Kirkland et al., 2007c)) and to evaluate the potential for heritable  
28 mutagenic effects. The *in vivo* genotoxicity testing strategy may also be  
29 required by regulatory authorities where there is a likelihood of high, or  
30 moderate and prolonged human exposure. Genotoxicity testing using animals  
31 should be carried out when there is no suitable alternative, and the minimum  
32 number of animals should be used, consistent with obtaining valid results. If

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1 feasible, studies can be conducted as an adjunct to single or repeat dose  
2 toxicity studies. The COM supports current and future developments to  
3 replace, refine or reduce the need for *in vivo* genotoxicity

4 **V. Strategy for the Assessment of Chemical Substances with Existing**  
5 **Genotoxicity Data**

6 19. The principles for genotoxicity testing and assessment of chemical substances  
7 which have existing but possibly limited and/or inadequate genotoxicity data  
8 are essentially similar to those required for a chemical substance which has no  
9 genotoxicity data available. They are based on Stages 0, 1 and 2 outlined  
10 above, although a step genotoxicity testing strategy needs to be taken on a  
11 case-by-case basis using the following steps (shown in Figure 1);

12 *Step 1* Consider the purpose of the testing strategy using one or more of the  
13 following objectives. 1) Screen for genotoxic potential. 2) Investigate  
14 genotoxicity in tumour target tissue(s), 3) Investigate potential for germ cell  
15 genotoxicity, 4) Investigate mutagenic end point(s) identified from available  
16 genotoxicity data.

17 *Step 2* Assess the available genotoxicity data and the adequacy of each study  
18 in order to reach conclusions on genotoxic potential (i.e. positive, negative or  
19 equivocal). Use available Structure Activity Relationship (SAR) approaches as  
20 an additional aid in the evaluation of genotoxicity tests. Such SAR data may  
21 also be helpful in identifying misleading genotoxicity test results.

22 *Step 3* Consider the weight of evidence that can be attributed to the  
23 genotoxicity results obtained and whether there is sufficient robust evidence to  
24 assess gene mutation, clastogenicity and aneugenicity. Use appropriate  
25 criteria to decide if any positive or equivocal results may be misleading and  
26 determine MoGA if appropriate.

27 *Step 4* If (from steps 1-3) adequate genotoxicity data are available then it is  
28 possible to derive conclusions on mutagenic hazard. If the available evidence  
29 is insufficient to reach conclusions on mutagenic hazard, identify key data  
30 gaps taking into account the purpose of the evaluation and derive a plan for  
31 each stage of the COM testing strategy as appropriate. This may include

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- 1 repeating specific genotoxicity tests from each stage of the COM testing  
2 strategy and/or undertaking additional core studies from Stages 1 and 2 as  
3 appropriate.
- 4 20. If (from steps 1-3) there are no genotoxicity data available, then publicly  
5 available (Q)SAR databases can provide a preliminary assessment of  
6 genotoxic potential (see guidance given below in paragraphs 23-27) and  
7 helpful information to aid in deciding priorities for genotoxicity testing. In  
8 addition the Threshold of Toxicological Concern (TTC) concept can also  
9 provide helpful information to aid in considering testing priorities. The TTC  
10 is an exposure level for any unstudied chemical (regardless of chemical class)  
11 that will not pose a risk of significant carcinogenicity. For genotoxic  
12 chemicals the TTC is set at the low exposure level of 1.5 µg/kg bw/day (Kroes  
13 et al., 2004). Dearfield and colleagues published a rationale (Dearfield et al.,  
14 2010) with regard to follow-up testing of positive *in vitro* mutagenic effects,  
15 which involves interpreting data, assigning a weight of evidence to specific  
16 genotoxicity data, and reaching decisions on data gaps in order to derive  
17 conclusions on further testing. This is a valuable guide to aid in decision  
18 making using the approach given above for chemicals with existing limited  
19 and/or inadequate genotoxicity data.

## 20 **VI Genotoxicity Testing Strategy**

- 21 21. The COM guidance provides a strategy for testing all chemical substances  
22 including those which have existing (and often limited or inadequate)  
23 genotoxicity data. Test substances may also contain impurities at varying  
24 levels which may also exhibit genotoxic activity. Separate guidance on the  
25 genotoxicity assessment of impurities has been identified as a priority project  
26 during the COM horizon scanning exercise in 2010 (see minutes of COM  
27 meeting of October 2010 <http://www.iacom.org.uk/meetings/index.htm>) . The  
28 strategy recommended in the following sections is concerned with testing for  
29 genotoxic activity of chemical substances and not with mixtures of chemicals.  
30 Since the publication of the COM guidance in 2000, assessments of the  
31 performance of SAR approaches, screening tests and genotoxicity assays (both  
32 individually and in combinations) regarding the prediction of rodent

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1 carcinogenicity have been published (Kirkland et al., 2005a, Matthews et al.,  
2 2006, Matthews et al., 2006, Kirkland and Speit, 2008). Reference to these  
3 publications can provide an insight into the performance of the genotoxicity  
4 assays specifically in relation to the particular data sets analysed and the end  
5 points considered (predominantly rodent carcinogenicity). Relevant  
6 sensitivity and specificity data and assay performance assessments have been  
7 summarised in Annex 1 for information and are cited where appropriate in the  
8 text below. Overall the available data suggest that mammalian cell assays for  
9 mutagenicity including the mouse lymphoma assay do not perform well at  
10 discriminating between rodent carcinogens and non-carcinogens.

### 11 **Stage 0: Preliminary Considerations Prior to Genotoxicity Testing**

12 22. The intrinsic chemical and toxicological properties of the test substance must  
13 be considered before devising the genotoxicity testing programme.

#### 14 Physico-chemical and Toxicological Properties

15 23. The physico-chemical properties of the test substance (for example, pKa,  
16 partition coefficient, solubility, and stability in solvents/vehicles) and its purity  
17 can affect the ease of conduct and results of *in vitro* tests. For example, the  
18 tolerance of cells to acidic chemicals can be enhanced by neutralisation but  
19 this may affect the inherent reactivity of substances to DNA (Hiramoto et al.,  
20 1997). Alternatively, low solubility may limit the feasibility of undertaking  
21 some or all of the *in vitro* mutagenicity tests recommended in this strategy.  
22 The potential for auto-oxidation of the test chemical in the culture medium can  
23 also affect the outcome of *in vitro* genotoxicity tests. It is noteworthy that the  
24 toxic properties of test substances, such as target organ effects, or  
25 irritancy/corrosivity in contact with skin or mucous membranes and their  
26 toxicokinetics and metabolism will influence the choice of route of  
27 administration and the highest dose level achievable in Stage 2 *in vivo*  
28 mutagenicity tests.

#### 29 Structure Activity Relationships

30 24. Whether the test substance would be expected to have mutagenic potential can  
31 be assessed from its chemical structure, which may provide structural alerts

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1 for mutagenicity. A composite model structure was originally devised by  
2 Ashby and Tennant in 1991 indicating substituent chemical groups or moieties  
3 associated with DNA-reactivity (Ashby and Paton, 1993). A number of freely  
4 available and commercial systems to investigate structure activity  
5 relationships (SAR) for mutagenicity have been developed and evaluated since  
6 2000 (Zeiger et al., 1996, Cariello et al., 2002, Contrera et al., 2005, Snyder  
7 and Smith, 2005, Benigni et al., 2007, Benigni and Bossa, 2008). The OECD  
8 (OECD., 2004) and the European Commission (Joint Research Centre) have  
9 published principles for the validation of (Q)SAR ((Quantitative) Structure  
10 Activity Relationships) (Worth et al., 2005, Benigni and Bossa, 2008).  
11 (Q)SAR assessment of the *in vitro* mutagenicity in bacteria has been attained  
12 by two types of approach; statistical analyses of structure and mutagenic  
13 activity and/or (Q)SAR models using programmed rules for prediction of  
14 mutagenic activity based on the available knowledge and expert judgement.  
15 An example of a ruled based approach is DEREK (Deductive Estimation of  
16 Risk from Existing Knowledge). This was developed by Lhasa Ltd,  
17 (<https://www.lhasalimited.org/derek/>) (Marchant and Group, 1996 ) The  
18 output from statistically based models is a quantitative probability for the  
19 endpoint under consideration. Examples include MultiCASE (Multiple  
20 Computer Automated Structure Evaluation; <http://www.multicase.com/>) and  
21 TOPKAT (Toxicity Prediction by Komputer Assisted Technology;  
22 <http://accelrys.com/mini/toxicology/predictive-functionality.html>). Some  
23 databases and models for prediction of *in vitro* mutagenicity (including  
24 bacterial and mammalian cell systems) have been developed by the European  
25 Chemicals Bureau (ECB) ([http://ecb.jrc.ec.europa.eu/qsar/qsar-](http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=QRF)  
26 [tools/index.php?c=QRF](http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=QRF)) and the US Food and Drugs Administration  
27 (<http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092221.htm>).  
28 25. The European Commission is funding the SCARLET (Structure-activity  
29 relationships leading experts in mutagenicity and carcinogenicity) project  
30 ([www.scarlet-project.eu/](http://www.scarlet-project.eu/)). A model for mutagenicity prediction (e.g  
31 CAESAR (Computer Assisted Evaluation of industrial chemical Substances  
32 According to Regulations) is freely available ([www.caser-project.eu](http://www.caser-project.eu))  
33 (Benfenati et al., 2009). A structural alert database for mutagenicity (Toxtree)

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1 is also freely available from the ECB internet site  
2 (<http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE>) (Benigni  
3 and Bossa, 2008). The OECD Toolbox includes software and data bases for  
4 use in the prediction of genotoxicity and is also freely available.  
5 ([http://www.oecd.org/document/23/0,3343,en\\_2649\\_34379\\_33957015\\_1\\_1\\_1\\_1,00.html](http://www.oecd.org/document/23/0,3343,en_2649_34379_33957015_1_1_1_1,00.html)) Information on approaches to the evaluation of model validity  
6 and the appropriate documentation for demonstrating model validity (the  
7 QSAR Model Reporting Format (QMRF)) has been published by the  
8 European Chemicals Agency (Guidance on Information Requirements and  
9 Chemical Safety Assessment Chapter R6  
10 [http://guidance.echa.europa.eu/docs/guidance\\_document/information\\_requirements\\_en.htm](http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm)). Information on completed QMRFs for (Q)SAR models can be  
11 accessed at <http://qsar.db.jrc.it/qmrf/>.  
12  
13  
14 26. Such (Q)SAR systems can be useful when a large number of chemicals require  
15 assessment and prioritisation for genotoxicity testing or in instances where a  
16 rapid assessment of a chemical is required and there are no genotoxicity test  
17 data available. Each (Q)SAR system has a defined domain of applicability  
18 which is determined by the structural/descriptor factors, modes/mechanism of  
19 mutagenicity, and metabolic aspects included within the system. In addition *in*  
20 *silico* approaches can aid in the interpretation of Stage 1 *in vitro* genotoxicity  
21 test results (Dearfield et al., 2010). The available systems perform well for  
22 prediction of bacterial mutagenicity (i.e. for chemical structures within the  
23 domain of applicability of the model under consideration) (see Annex 1).  
24 However, lower sensitivities and specificities have been reported for a number  
25 of systems when used for prediction of results from *in vitro* cytogenetics or  
26 the mouse lymphoma assay (e.g. using MCASE and MDL-QSAR) (Contrera  
27 et al., 2008). One factor in the lower predictive capability of (Q)SAR  
28 systems for mammalian cell genotoxicity assays is inadequate coverage of  
29 non-covalent DNA interactions and non-DNA targets associated with cell  
30 division (Grant et al., 2000, Snyder and Smith, 2005). It has also been  
31 proposed that (Q)SAR assessments can aid in the interpretation of the  
32 relevance of *in vitro* genotoxicity assays through prediction of  
33 biotransformation (Combes et al., 2007). Other systems combining metabolic

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1 simulation with structure toxicity rules have been developed (e.g TIMES;  
2 tissue metabolic simulator) but are at a relatively early stage of validation  
3 (Mekenyan et al., 2004, Serafimova et al., 2007). Lhasa Ltd have developed a  
4 computer programme (METEOR), which has the facility to integrate  
5 prediction of metabolism with (Q)SAR approaches for genotoxicity.  
6 (<https://www.lhasalimited.org/meteor/>)

7 27. An authoritative and comprehensive evaluation of the different (Q)SAR  
8 approaches to the identification of genotoxic potential has been prepared for  
9 European Food Safety Authority (EFSA)  
10 (<http://www.efsa.europa.eu/en/scdocs/scdoc/50e.htm>) by the Computational  
11 Toxicology group, Institute for Health & Consumer Protection, European  
12 Commission-Joint Research Centre (JRC), Ispra, Italy. A dataset comprising  
13 pesticides, the Distributed Structure-Searchable Toxicity (DSST) database and  
14 EU classified mutagens was used to assess a wide range of computer based  
15 predictive models. Overall the JRC concluded the (Q)SAR approaches  
16 reviewed were shown to produce acceptable results for the prediction of  
17 bacterial mutagenicity and that the use of a two-software combination  
18 (including assessment of (Q)SAR data on structural analogues of the chemical  
19 under consideration) can reduce the false negative rate for the identification of  
20 classified mutagens. A combination of CAESAR and Toxtree yielded a false  
21 negative prediction rate of 11% for classified mutagens.

22 28. A compilation of structural alerts for prediction of the rodent *in vivo*  
23 micronucleus assay has recently been published. The authors advocate that the  
24 derived rules can be used for preliminary identification of *in vivo* mutagens  
25 (Benigni et al., 2010).

26 29. Overall, (Q)SAR approaches for the prediction of genotoxic activity can be a  
27 valuable tool to aid in the high throughput screening of compounds, the  
28 provision of assessments for chemicals for which no genotoxicology test data  
29 are available and also prioritisation for genotoxicity testing. (Q)SAR can also  
30 aid in the interpretation of genetic toxicology tests, although currently such  
31 predictions cannot replace the need to undertake the *in vitro* and *in vivo*  
32 genotoxicity tests currently required to derive conclusions on mutagenic

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1 hazard. With regard to chemicals for which there are limited, possibly  
2 inadequate, or no genotoxicity test data available, a (Q)SAR prediction of  
3 mutagenicity (within the domain of applicability of the system(s) used) should  
4 be taken as preliminary evidence for potential or lack of potential  
5 mutagenicity. However, expert judgement is needed when reaching  
6 conclusions on mutagenic hazard on the basis of (Q)SAR information alone.  
7 In reaching conclusions, data from well conducted *in vitro* genotoxicity tests  
8 should be attributed a much higher weight of evidence than (Q)SAR  
9 predictions, although all information should be assessed on a case-by-case  
10 basis.

### 11 Screening Tests

12 30. There are a number of current initiatives which attempt to combine data  
13 mining *in silico* approaches with high throughput tests to develop approaches  
14 to screening large numbers of novel chemicals (Benfenati et al., 2009.). In this  
15 guidance, genotoxicity screening tests refers to high throughput tests which  
16 have been designed to be rapid, economical, reproducible, require only small  
17 amounts of test substances (typically below 50 mg) and have a high  
18 concordance with comparator genotoxicity end points in genotoxicity tests.  
19 (These tests are also often referred to as pre-screening tests.) High throughput  
20 bacterial tests have been developed using combinations of *Salmonella* tester  
21 strains (Ames II), primary DNA damage (*umu* assay), mutations in  
22 ampicillinase gene (MutaGen assay), bioluminescence or 5-fluorouracil  
23 resistance (Reifferscheid et al., 2005, Miller et al., 2005 , Aubrecht et al.,  
24 2007, Kamber et al., 2009 , Ackerman et al., 2009.). Other screening systems  
25 cited in the literature include DNA repair activity in yeast cells (Westerink et  
26 al., 2009). One research group has proposed a combination of two commercial  
27 screening assays (Vitotox<sup>TM</sup> for bacterial mutagenicity and RadarScreen yeast  
28 screen for clastogenicity) for rapid screening of compounds. (Westerink et al.,  
29 2009).

30 31. A number of genotoxicity screening tests using *in vitro* systems have been  
31 proposed including, alkaline elution using rat hepatocytes (Gealy et al., 2007),  
32 the detection of DNA damage (via p53 or GADD45a activation, GreenScreen)

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1 in cell lines (Knight et al., 2009) and differential growth in DNA repair  
2 proficient and deficient cell lines (Helleday et al., 2001). A screening test for  
3 genotoxicity using HepG2 (metabolically competent with wild type p53  
4 genotype) based on four different luciferase-reporter assays has been  
5 published. The authors claim, based on a small dataset, a high sensitivity for  
6 prediction of genotoxicity when used in combination with the commercially  
7 available systems (Vitotox<sup>TM</sup> and RadarScreen) (Westerink et al., 2010).  
8 None of these genotoxicity screening tests have reached the stage of  
9 development where they could routinely be used to replace data generated  
10 from *in vitro* genotoxicity testing. The predominant use of high throughput  
11 screening tests is as an aid in prioritisation of compounds for development  
12 undertaken by industry. The Committee has agreed that the GADD45a-GFP  
13 assay is most suited as part of a battery of high throughput screening (COM  
14 minutes March 2010, <http://www.iacom.org.uk/meetings/index.htm>).

15 32. High throughput genotoxicity screening tests can be used in a tiered approach  
16 with *in vitro* genotoxicity tests during chemical development. It has been  
17 suggested that greater validation and acceptance by regulatory authorities of  
18 these tests could lead to the replacement of existing genotoxicity testing  
19 strategies with a combination of high throughput screening tests (Custer and  
20 Sweder, 2008).

## 21 **Stage 1: *In Vitro* Genotoxicity Testing (Figure 2)**

### 22 Overview of strategy

23 33. The COM concluded in 1989 and 2000 that it was appropriate to concentrate  
24 on a relatively small number of assays, using validated, sensitive methods  
25 particularly chosen to ***avoid misleading negative results***. Two important parts  
26 of the revised Stage 1 strategy include using appropriate tests to gain an  
27 insight into the nature of the genotoxic effects of a test substance and to ***avoid***  
28 ***misleading positive results***. Misleading positive results have been reported for  
29 certain mammalian cell assays (Kirkland et al., 2007a, Pfuhler, 2009)(Fowler  
30 et al., 2009a).

31 34. As outlined above in paragraph 13, Stage 1 involves tests for genotoxic  
32 activity using *in vitro* methods and comprises a two test core system (namely

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1 an Ames test and *in vitro* micronucleus test (MNvit)) with the objective of  
2 assessing mutagenic potential by investigating three different end points (gene  
3 mutation, structural chromosomal damage and changes in chromosome  
4 number). A clear positive result in either of these two core tests is sufficient to  
5 define the chemical as an *in vitro* mutagen, although further *in vitro* and/or *in*  
6 *vivo* testing may be undertaken to understand the relevance of the positive  
7 results. The Committee considers this revised strategy allows for efficient  
8 identification of all mutagenic end points with an optimal low level of  
9 misleading positive results. The rationale for this is given in Annex 3.  
10 Additional investigations of chemicals which give positive or repeated  
11 equivocal results in Stage 1 tests can include an assessment of mode(s) of *in*  
12 *vitro* genotoxic action (MoGA). There are a number of reasons (discussed  
13 below) why positive results in *in vitro* genotoxicity tests might occur by  
14 mode(s) of action not relevant to human health hazard assessment. Such  
15 MoGA evaluation *in vitro* is particularly relevant for those chemicals (e.g.  
16 cosmetics) where there is a regulatory constraint which precludes the use of *in*  
17 *vivo* genotoxicity assays in the testing strategy. The COM does not  
18 recommend the use of *in vitro* genotoxicity assays that have not been cited in  
19 this guidance such as assays for sister chromatid exchange, the *in vitro* UDS  
20 assays or tests using fungi. A table of mutagenic endpoints detected by each  
21 genotoxicity assay cited in Stage 1 of this strategy is given in Annex 2.

22 35. For chemicals which give equivocal results or repeated small positive effects  
23 it is important to consider evidence of reproducibility, and the magnitude of  
24 the induced genotoxic effect in relation to historical negative control data and  
25 then consider whether further *in vitro* genotoxicity testing is needed (Kirkland  
26 et al., 2007b, Hayashi et al., 2010). Further consideration of MoGA and SAR  
27 data for these chemicals can also give valuable information (Dearfield et al.,  
28 2010).

29 36. In general, if clear negative results are obtained in all *in vitro* tests undertaken,  
30 it can be concluded that the chemical has no mutagenic activity. However,  
31 there are some occasions when additional *in vitro* genotoxicity testing may be  
32 undertaken for chemicals giving a negative response in the two *in vitro* core

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1 genotoxicity tests for example where tumours are subsequently found in  
2 rodents and specific rodent or human metabolites need to be subject to  
3 genotoxicity assessment, or the test substance has a structural alert but *in vitro*  
4 genotoxicity tests were negative. A further testing strategy would have to be  
5 designed on a case-by-case basis (Muller et al., 2003a, Kirkland et al., 2007b).  
6 An IWGT working group has published guidance on this topic (Kasper et al.,  
7 2007). An important part of any additional *in vitro* strategy should be  
8 consideration of the appropriate exogenous metabolic activation system  
9 (including alternative sources of S-9 or other metabolic systems including  
10 genetically engineered cell lines)(Ku et al., 2007b). Further information on *in*  
11 *vivo* genotoxicity testing of such test substances is provided in Stage 2 of this  
12 strategy.

13 37. Information from other combinations of genotoxicity tests which may include  
14 one or more non-core tests outlined below in paragraphs 57-59 (and in Figure  
15 2) may also give adequate data on all three end-points on a case-by-case basis.  
16 *In vitro* genotoxicity tests using human reconstructed skin may provide useful  
17 information on *in vitro* mutagenic hazard in circumstances where *in vivo*  
18 testing is not permitted, for example with cosmetic ingredients.

19 38. The full Stage 1 strategy should be performed and the results of studies  
20 evaluated before a decision is made on whether to proceed to Stage 2 testing  
21 or whether a conclusion on mutagenic hazard can be derived for test  
22 substances where no *in vivo* genotoxicity testing is permitted. An outline of  
23 Stage 0 and Stage 1 (*in vitro* genotoxicity testing) is given in Figure 2 and a  
24 description of the assays recommended is provided in the following  
25 paragraphs.

#### 26 Discussion of Stage 1 Tests- General Aspects

27 39. The conduct of genotoxicity assays has improved over time and the overall  
28 sensitivity of *in vitro* testing strategies regarding prediction of rodent  
29 carcinogens is very high(Kirkland et al., 2007a). Proposals have been  
30 published for genotoxicity testing advocating a single *in vitro* genotoxicity test  
31 (Ku et al., 2007a) or a complex approach involving up to six *in vitro*  
32 genotoxicity tests (Kirkland et al., 2005b). Neither of these approaches is

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1 considered preferable to the proposed Stage 1 core testing. Although the  
2 sensitivity of the Stage 1 tests is very high, the specificity (producing negative  
3 results with non-carcinogens) is poor (Kirkland et al., 2007d). Possible  
4 reasons for the poor specificity have been discussed by various working  
5 groups e.g. see Kirkland et al 2007d. A comprehensive review of the  
6 performance of Stage 1 genotoxicity assays for prediction of rodent  
7 carcinogenicity reported positive results in one or more *in vitro* tests for a  
8 substantial number of rodent non-carcinogens (as assessed by the  
9 Carcinogenic Potency Database (CPDB), National Toxicology Program  
10 (NTP), and the International Agency for Research on Cancer (IARC)). Thus  
11 the specificity (i.e. correct identification of rodent non-carcinogens) was  
12 considered to be reasonable for the Ames test (74%) but poor for the  
13 mammalian cell assays (below 45%) (Kirkland et al., 2005a).

14 40. Data on the sensitivity, including the combination of Ames test and *in vitro*  
15 micronucleus test for the detection of rodent carcinogens and *in vivo*  
16 genotoxins, are given in Annex 1. It is difficult to draw precise conclusions  
17 from these data since the databases of chemicals used vary. However these  
18 data do show that mammalian cell genotoxicity tests have low specificity and  
19 that combinations of *in vitro* genotoxicity tests result in high sensitivity. A  
20 high sensitivity has been a priority of previous genotoxicity testing strategies  
21 recommended by the COM (DOH., 2000). An evaluation of the use of *in vitro*  
22 genotoxicity tests to predict rodent carcinogens and *in vivo* genotoxins  
23 prepared for the COM meeting in June 2010  
24 (<http://www.iacom.org.uk/papers/index.htm> MUT/2010/08) concluded that  
25 there is no convincing evidence that any rodent carcinogen or *in vivo*  
26 genotoxin would be “missed” by using an *in vitro* genotoxicity test battery  
27 consisting of Ames test and *in vitro* micronucleus test. Further rationale and  
28 justification for using this combination of *in vitro* tests are provided in Annex  
29 3.

30 41. It is most likely that the few occasions where *in vitro* test strategies fail to  
31 detect mutagenic activity (i.e. misleading negative results) will be due to the  
32 absence of appropriate metabolic activity *in vitro* (Brambilla and Martelli,

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1           2004). Approaches to resolving potential inadequacies in metabolic activation  
2           include structure based metabolism predictions, use of genetically modified  
3           target organisms (e.g. CYP2E1 in *Salmonella* YG7108pin3ERb<sub>5</sub>) (Emmert et  
4           al., 2006), the use of exogenous metabolic activation systems derived from  
5           human sources, or recombinant human cytochrome P450 systems as an  
6           external activation system (Ku et al., 2007b).

7   42.    There are a number of non-DNA interaction MoGAs by which a chemical may  
8           demonstrate an *in vitro* genotoxic effect that is either not relevant for humans  
9           or has a threshold. In both cases a No Observed Effect Concentration (NOEC)  
10          can be determined and may be useful in evaluating risk. The COM has  
11          reviewed the evidence for a number of threshold MoGAs and a general  
12          guidance statement is available (<http://www.iacom.org.uk/guidstate/index.htm>  
13          statement G05). Threshold MoGAs can be generally be considered as an  
14          overload of normal cellular physiology'. Investigations of MoGA need to be  
15          designed on a case-by-case basis and can be complex to interpret (Kirkland et  
16          al., 2007a).

17   43.    There has been considerable debate regarding the highest concentration that  
18           should be used routinely in mammalian cell assays. The International  
19           Conference on Harmonisation of the Technical Requirements for Registration  
20           of Pharmaceuticals for Human Use (ICH) is considering whether the  
21           maximum concentration tested for pharmaceuticals should be 1mM in  
22           mammalian cell genotoxicity assays which would have the effect of reducing  
23           the number of misleading positive results due to excessive concentrations  
24           where the cellular defence mechanisms might be overwhelmed. However a  
25           reduction to 1mM would not be consistent with the OECD recommendation  
26           for a top concentration of 10mM in mammalian cell genotoxicity assays  
27           (OECD., 1997). A recent analysis of published data for the top concentration  
28           in mammalian cell genotoxicity tests identified a small number of carcinogens  
29           that (according to the publications) would not be detected in any part of a  
30           three test *in vitro* genotoxicity test battery (consisting of the Ames, mouse  
31           lymphoma and *in vitro* chromosomal aberration tests) if the testing  
32           concentration limit for mammalian cell assays were reduced from 10mM to

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1 1mM (Parry et al., 2010) A further investigation of these carcinogens found  
2 that some positive results at concentrations above 1mM were not reproducible  
3 (i.e. they were not genotoxic in mammalian cells under current OECD  
4 guideline protocols) and others were positive at concentrations below 1mM,  
5 particularly when continuous treatments in the absence of S-9 (not included in  
6 the original publications) were conducted. Thus a new upper limit for  
7 mammalian cells tests of 1mM or 500 µg/ml (whichever is higher) has been  
8 proposed as sufficient to detect all genotoxic carcinogens that are negative in  
9 the Ames test (Kirkland and Fowler, 2010). Thus the available evidence  
10 supports an upper concentration limit of 1mM for mammalian cells but there is  
11 a need to reach international consensus on this proposal before making a  
12 recommendation on its application to genotoxicity tests using mammalian  
13 cells.

14 44. There has also been considerable investigation of the role of excessive  
15 cytotoxicity in mammalian cells and choice of cell type as possible causes of  
16 misleading positive results (Blakey et al., 2008, Fellows et al., 2008b, Pfuhler,  
17 2009). The method used to assess cytotoxicity may affect the selection of  
18 highest concentration tested and potentially the results obtained using  
19 mammalian cell genotoxicity assays (Kirkland et al., 2007d). However, it is  
20 important to note that although excessive cytotoxicity may lead to misleading  
21 positive results, it may also result in misleading negative results when  
22 pronounced cell cycle delay occurs. A similar conclusion was reached at an  
23 international symposium on regulatory aspects of genotoxicity testing (Blakey  
24 et al., 2008)). Many cell lines used for genotoxicity testing lack appropriate  
25 metabolism leading to reliance on exogenous metabolic activation systems.  
26 These cell lines also have impaired p53 function and altered DNA repair  
27 capacity (Kirkland et al., 2007d). There is some evidence that human  
28 lymphocytes are less susceptible to misleading positives than the rodent cell  
29 lines currently used (e.g. CHO, V79 CHL and V79). Other cell systems such  
30 as the human cell lines HepG2, TK6 and MCL-5 cells and the reconstructed  
31 human skin models show promise for future use (Kirkland et al., 2007d,  
32 Fowler et al., 2009b). The COM agrees that it is not necessary to undertake

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1 independent confirmatory *in vitro* tests when clear negative or positive results  
2 have been obtained provided the following criteria are satisfied:

- 3 • there is no doubt as to the quality of the study design and the conduct  
4 of the test,
- 5 • the spacing and range of test substance concentrations rule out missing  
6 a positive response,
- 7 • sufficient treatment, conditions and sampling times have been used
- 8 • the result is neither clearly negative nor clearly positive (i.e. is  
9 considered to be equivocal) by appropriate statistical and biological  
10 criteria.

11 45. It is recognised that it can be difficult to provide convincing evidence for an  
12 absence of a genotoxic effect (i.e. a consistently clear negative result). The  
13 investigator should consider the power of the study design and the past  
14 performance of the test system when formulating a protocol in order to  
15 optimise the chances of obtaining an unequivocal result from a single  
16 experiment and to ensure that any potential genotoxic effect is not missed.

17 46. There is a need to undertake further *in vitro* genotoxicity testing when an  
18 equivocal result is obtained. Such additional genotoxicity tests need to be  
19 planned on a case-by-case basis and need not necessarily be undertaken in an  
20 identical fashion to the initial experiment(s). Indeed it may be preferable to  
21 alter certain aspects of the study (e.g. concentration levels investigated,  
22 treatment and sampling times, concentration of metabolic activation mix) so as  
23 to obtain supplementary data. It may also be appropriate to use a different  
24 genotoxicity test system, e.g. a chromosomal aberration test, if there is  
25 equivocal evidence of clastogenicity from an *in vitro* micronucleus test, or a  
26 mouse lymphoma mutation assay if there is equivocal evidence of gene  
27 mutations from an Ames test.

28 47. The use of historical negative control data to aid in the interpretation of  
29 genotoxicity test results has been considered particularly in relation to  
30 equivocal and small magnitude genotoxic effects (Kirkland et al., 2007b).  
31 Advice has been recently published on approaches to collecting historical

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1 control data. Ideally data should be reported in terms of means and confidence  
2 intervals for the distribution of baseline genotoxic effects rather than observed  
3 ranges where outliers can have a disproportionate effect. The dataset should  
4 be updated regularly and should be as large as possible. Historical negative  
5 control data should have been generated using a fixed testing protocol unless it  
6 can be demonstrated that changes in protocol do not impact on the range of  
7 values reported in studies (Hayashi et al., 2010). The Committee considers  
8 that negative historical control data can be a valuable aid in the interpretation  
9 of genotoxicity tests.

10 48. If a chemical is considered on the basis of Stage 1 genotoxicity test results to  
11 have *in vitro* mutagenic potential but has not been tested *in vivo*, the COM  
12 considers it prudent to assume that the substance may have *in vivo* mutagenic  
13 potential.

#### 14 Discussion of Stage 1 strategy.: Specific Core Tests

##### 15 *In Vitro Bacterial Tests for Gene Mutations*

16 49. The most widely used *in vitro* mutagenicity test is the bacterial reverse  
17 mutation assay for gene mutations developed by Ames and his colleagues  
18 using *Salmonella typhimurium* (Gatehouse et al., 1994) The very extensive  
19 database available for this assay justifies its inclusion in any initial  
20 genotoxicity testing for mutagenic hazard. Several strains of bacteria capable  
21 of detecting both base-pair and frame-shift mutations must be included, the  
22 best validated strains being TA 1535, TA1537 (or TA97 or TA97a), TA98 and  
23 TA100. These strains of *Salmonella typhimurium* may not detect some  
24 oxidising mutagens and cross linking agents and thus *Escherichia coli* WP2  
25 (pKM101), WP2*uvrA* or *Salmonella* TA102 should also be used. Testing  
26 should be carried out both in the presence and absence of an appropriate  
27 exogenous metabolic activation system.

28 50. There have been developments to automate and minimise the amount of test  
29 substance required for the Ames test (e.g. Spiral *Salmonella* mutagenicity  
30 assay (Claxton et al., 2001) and Ames II<sup>TM</sup> test (Fluckigettr-Isler et al., 2004)).  
31 These methods have not been developed to a point where they can be routinely  
32 be used for regulatory submissions.

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1 *In Vitro Mammalian Cell Micronucleus Assay (MNvit) for Clastogenicity and*  
2 *Aneuploidy*

3 51. The COM recommended in 2000 that equivalent information on clastogenicity  
4 and aneuploidy could be obtained from the *in vitro* micronucleus assay  
5 (MNvit) compared with chromosomal aberration testing in mammalian cells  
6 (metaphase analysis) but that aneuploidy could be more easily detected by  
7 MNvit. This has since been confirmed in a collaborative trial (Lorge et al.,  
8 2006). The COM was aware in 2000 of the ongoing protocol developments  
9 and validation of this assay but noted that development of an OECD guideline  
10 would take some time. Since 2000 there have been extensive and authoritative  
11 investigations of the utility of the *in vitro* micronucleus assay, and an  
12 ECVAM (European Centre for the Validation of Alternative Methods)  
13 retrospective validation study concluded that the MNvit is reliable and can be  
14 used as an alternative to the *in vitro* chromosomal aberration assay (Corvi et  
15 al., 2008). OECD guideline 487 has now been adopted  
16 (<http://www.oecd.org/dataoecd/38/58/39780112.doc>). Many current published  
17 *in vitro* genotoxicity testing strategies recommend that the micronucleus assay  
18 and metaphase analysis can be considered as equivalent in the detection of  
19 clastogens (Cimino, 2006, Eastmond et al., 2009). However the detection of  
20 aneugens in the metaphase test requires non-standard approaches and the  
21 COM recommends the *in vitro* micronucleus assay as the first choice test for  
22 clastogenicity and aneuploidy detection.

23 52. The *in vitro* micronucleus test can be carried out in the absence or presence of  
24 cytochalasin B, which is used to block cell division and generate binucleate  
25 cells (CBMN method). The advantage of using cytochalasin B is that it allows  
26 clear identification that treated and control cells have divided *in vitro* and  
27 provides a simple assessment of cell proliferation. The use of cytochalasin B  
28 has no impact on the sensitivity of the test results (Garriott et al., 2002, Lorge  
29 et al., 2006, Oliver et al., 2006, Wakata et al., 2006). Micronuclei are scored  
30 in mononucleated cells when experiments are performed in the absence of  
31 cytochalasin B. The target population in the presence of cytochalasin B is the  
32 binucleate cells (because it is clear they have divided), however scoring of

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1 both mononucleated and binucleated cells can be useful for the detection of  
2 aneuploids (Lorge et al., 2006, Wakata et al., 2006).

3 53. There have been major international collaborative investigations to develop  
4 the protocol (Garriott et al., 2002, Phelps et al., 2002, Kirsch-Volders et al.,  
5 2003, Lorge et al., 2006), provide information on the performance of this  
6 assay using different cell lines (Oliver et al., 2006, Wakata et al., 2006,  
7 Fowler, 2009, Pfuhler, 2009), to investigate the most appropriate methods for  
8 measuring cytotoxicity (Fellows et al., 2008a, Lorge et al., 2008, Kirkland,  
9 2010) and initial studies to evaluate a flow cytometric approach to the  
10 micronucleus assay (Bryce et al., 2007, Bryce et al., 2008a, Laingam et al.,  
11 2008). The *in vitro* micronucleus assay can be performed using most  
12 mammalian cell lines used in genotoxicity testing (Lorge et al., 2006).  
13 However there is emerging evidence that rodent cell lines with compromised  
14 p53 activity such as V79, CHO and CHL cells can give more misleading  
15 positive results than cell lines proficient for p53 activity such as TK6, HepG2  
16 and human lymphocytes (Fowler et al., 2009a). Overall the COM's preference  
17 is for human lymphocytes which have a number of advantages over cell lines  
18 (e.g. normal diploid primary human cells with some protection against  
19 oxidative damage when whole blood cultures are used).

20 54. There have been considerable developments on deriving suitable protocols for  
21 the *in vitro* micronucleus assay using both cell lines and lymphocytes (Garriott  
22 et al., 2002, Phelps et al., 2002, Kirsch-Volders et al., 2003, Aardema et al.,  
23 2006, Clare et al., 2006). One particular area of protocol development which  
24 has been subject to considerable investigation is the most appropriate  
25 method(s) for estimating cytotoxicity in *in vitro* micronucleus tests (Fellows et  
26 al., 2008a, Lorge et al., 2008, Kirkland, 2010). It has been suggested that  
27 using relative cell counts (RCC) may underestimate cytotoxicity and lead to  
28 potentially misleading positive results (Fowler et al., 2009b). In the absence  
29 of cytokinesis block, the relative increase in cell count (RICC) or relative  
30 population doubling (RPD) are comparable with replication index (RI) used  
31 with the cytokinesis block assay and are the most appropriate methods of  
32 cytotoxicity estimation. It has been suggested that testing beyond 50%

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1 survival is not necessary to identify potential mutagens (Fellows et al., 2008b,  
2 Lorge et al., 2008). However consensus recommendations embedded in the  
3 OECD guideline 487 indicate that the target range for cytotoxicity in the *in*  
4 *vitro* micronucleus test is 55±5%. Careful selection of toxicity measure has  
5 been shown to reduce the potential for misleading positive results (Fowler et  
6 al., 2009b).

7 55. The *in vitro* micronucleus assay can be combined with centromere or  
8 kinetochore stains, with pancentromeric or chromosome specific centromeric  
9 probes using fluorescence *in situ* hybridisation (FISH) as a sensitive way to  
10 discriminate between chromosome breaks, chromosome loss and chromosome  
11 non-disjunction and polyploidy (Kirsch-Volders et al., 2002) and therefore is  
12 useful in assessing mode of action (Parry, 2006). Binucleate cells obtained  
13 with the cytokinesis block methodology (CBMN) will usually be needed for  
14 determination of non-disjunction of chromosomes between daughter nuclei.  
15 Fenech has proposed that the CBMN assay can be further modified to provide  
16 comprehensive information on nucleoplasmic bridges (NPBs) which may  
17 provide information on chromosome rearrangements or telomere end fusions,  
18 and nuclear buds (NBUDs) which may provide information on gene  
19 amplification (Fenech, 2006, 2007). Fenech proposed that the comprehensive  
20 CBMN assay should be considered as a ‘cytome’ method for measuring  
21 chromosomal instability and altered cellular viability (Fenech, 2006). The  
22 ‘cytome’ method is complex and requires considerable technical skill and is  
23 currently not suitable for routine testing of chemicals for genotoxicity.

24 56. The flow-cytometry-based micronucleus assay (FCMMN) has the potential for  
25 increased reproducibility and decreased turnaround time for the micronucleus  
26 test (Laingam et al., 2008). However the potential still exists for misleading  
27 positive results from cell processing or from chemical induced apoptosis and  
28 necrosis (Laingam et al., 2008). Approaches to overcoming potential  
29 misleading positive results have included: the use of differential staining of  
30 micronuclei (MN) and necrotic and apoptotic cells, (Bryce et al., 2007, Bryce  
31 et al., 2008a), the use of electronic gating procedures and the use of  
32 concurrent assessment of cytotoxicity (Laingam et al., 2008). The FCMMN

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1 assay has also been adapted to cell lines which attach to solid surfaces (Bryce  
2 et al., 2010). The COM considers that further validation of the FCMMN assay  
3 is required before it can be used for regulatory submissions. A separate  
4 approach to automation of the CBMN assay involves automated image  
5 analysis using Giemsa stained slides (Decordier et al., 2009) which may be  
6 useful with appropriate validation.

#### 7 Discussion Stage 1: Non-Core Tests

##### 8 *In Vitro Chromosomal Aberration Assay in Mammalian Cells (Metaphase Analysis)* 9 *For Clastogenicity and Aneuploidy*

10 57. The *in vitro* chromosome aberration assay in mammalian cells has been widely  
11 used in genotoxicity testing for many decades. Only limited information can  
12 be obtained on potential aneugenicity by recording the incidence of polyploidy  
13 and/or modification of mitotic index (Aardema et al., 1998). The COM notes  
14 that polyploidy may not be a reliable indicator for aneugenicity and may result  
15 from a number of different genetic changes (Mitchell et al., 1995 , Galloway,  
16 2000) However the *in vitro* chromosomal aberration assay may provide  
17 information on exchanges which are associated with adverse health outcomes.  
18 It is important to include the use of chromosome specific centromeric probes  
19 with fluorescence in situ hybridisation (FISH) to assess the potential for  
20 aneuploidy. A wide range of FISH technologies exist for the analysis of  
21 clastogenic and aneugenic chromosome changes (Maierhofer et al., 2002).  
22 One published evaluation of *in vitro* genotoxicity testing strategies reported  
23 that there was no scientific basis for including both a chromosomal aberration  
24 and micronucleus assay in addition to Ames and mouse lymphoma assays  
25 (Kirkland et al., 2005b). An IWGT working group (Galloway et al., 2010) has  
26 agreed that the preferred measure of cytotoxicity in the chromosomal  
27 aberration test should be one based on cell proliferation (e.g. relative  
28 population doubling or relative increase in cell counts) in negative control  
29 cultures rather than simple cell counts. The available data indicate that the *in*  
30 *vitro* metaphase analysis and the *in vitro* micronucleus assay have similar  
31 overall performance as part of a strategy for genotoxicity testing. On balance

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1 it is considered preferable to use the *in vitro* micronucleus test for the  
2 assessment of clastogenic and aneugenic potential.

### 3 *In Vitro Mouse Lymphoma Assay for Gene Mutation*

4 58. The COM reaffirms the view stated in the 1989 and 2000 guidance, that the  
5 preferred *in vitro* mammalian cell gene mutation test is the mouse lymphoma  
6 assay. Certain mammalian cell gene mutation protocols that have been widely  
7 used, particularly some involving the use of Chinese hamster cells, are  
8 considered to be insufficiently sensitive for the identification of mutagens,  
9 predominantly on statistical grounds (UKEMS., 1989).

10 59. Since 2000, there has been considerable development of suitable protocols,  
11 negative solvent control data, criteria to define an acceptable positive control  
12 response and the use of the Global Evaluation Factor (GEF) and statistical  
13 analysis of test results (Clements, 2000, Moore et al., 2003, Kirkland et al.,  
14 2007c, Moore et al., 2007). Many of the published studies were undertaken by  
15 the US National Toxicology Program (NTP) and a recent re-evaluation of  
16 these results shows many of the studies to be uninterpretable or the outcomes  
17 to be equivocal (Schisler et al., 2010). Some authors have reported that the  
18 mouse lymphoma assay can detect, in addition to gene mutations and  
19 clastogenicity, information on recombination, deletion and aneuploidy (Ogawa  
20 et al., 2009, Wang et al., 2009). It is possible that aneuploidy in these cells  
21 could be a secondary effect of chromosomal rearrangement. However the  
22 COM considers that this assay is not appropriate for the routine assessment of  
23 aneuploidy.

### 24 *In Vitro Assays using Human Reconstructed Skin*

25 60. A number of research groups have developed genotoxicity assays based on  
26 micronuclei measurement using commercial sources of human reconstructed  
27 skin (such as Episkin<sup>®</sup> and EpiDerm<sup>™</sup>) (Curren et al., 2006, Flamand et al.,  
28 2006, Hu et al., 2009, Mun et al., 2009) or a co-culture technique involving  
29 reconstructed skin and mouse lymphoma L5178Y cells (Flamand et al., 2006).  
30 Proposals for the measurement of DNA damage using the comet assay in  
31 reconstructed skin have also been made (Pfuhler, 2009). The primary purpose  
32 in developing genotoxicity tests using reconstructed skin has been to

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1 supplement genotoxicity data-packages for cosmetic chemicals where no *in*  
2 *vivo* genotoxicity tests are permitted. A tiered approach to testing cosmetic  
3 ingredients for genotoxicity has recently been published (Pfuhrer et al., 2010).

#### 4 *In Vitro Alkaline Comet Assay for DNA Damage*

5 61. The *in vitro* alkaline comet assay for DNA damage has been proposed as an  
6 alternative to clastogenicity assessment in mammalian cells since cell  
7 proliferation is not needed, therefore any cell type can be used, and the assay  
8 results in fewer misleading positive results due to cytotoxicity or precipitation  
9 than chromosomal aberration tests (Hartmann et al., 2001, Witt et al., 2007).  
10 The alkaline comet assay detects a wide range of genetic damage including  
11 single and double strand breaks, repair induced breaks, alkali labile lesions and  
12 abasic sites. There is evidence that the *in vitro* comet can be used to detect  
13 DNA cross-linking agents (Spanswick et al., 2010). The comet-FISH assay  
14 has been recently developed to provide information on site specific DNA  
15 strand breaks (Glei et al., 2009). There is evidence that the *in vivo* comet  
16 assay can detect substances that induce gene mutations *in vitro* (Kirkland and  
17 Speit, 2008). Extrapolation from this would suggest that the *in vitro* comet  
18 assay can also detect substances that induce gene mutations. However it is  
19 not recommended as a replacement for gene mutation tests *in vitro*. Thus the  
20 comet assay measures DNA damage irrespective of MoGA, with the exception  
21 of aneuploidy. A positive comet assay result may be due to repairable DNA  
22 damage or lesions which lead to cell death and not necessarily mutations or  
23 micronuclei. Negative results from an Ames test and MNvit would reduce the  
24 level of concern associated with positive results from an *in vitro* comet assay.  
25 Thus, the *in vitro* comet assay can serve as a very useful adjunct to the  
26 recommended core-tests, especially in instances where *in vivo* testing is not  
27 permitted such as in cosmetic testing. However, since the comet assay does  
28 not detect aneuploidy, and may report repairable DNA damage, it is not  
29 recommended as a core *in vitro* test.

#### 30 **Summary Stage 1 (*In Vitro* Genotoxicity Testing)**

31 62. The COM recommendations for Stage 1 testing incorporate a number of  
32 changes to the 2000 guidelines, the main changes being the replacement of the

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1           *in vitro* metaphase analysis in mammalian cells with the *in vitro* micronucleus  
2           assay and a reduction from three tests to two *in vitro* tests for Stage 1. Tests  
3           should be undertaken according to the best international guidance available to  
4           avoid misleading positive or negative results. Data should be interpreted using  
5           appropriate statistical analysis and use of historical negative control data. The  
6           COM confirms the need to provide information on gene mutation,  
7           clastogenicity and aneugenicity in order to understand genotoxic mode(s) of  
8           action (MoGA) and to derive conclusions regarding the biological importance  
9           of results. Data on MoGA are important in elucidating whether genotoxicity  
10          tests give misleading negative or positive results, and also to aid decisions  
11          with regard to devising a strategy for Stage 2 *in vivo* genotoxicity testing.  
12          There is a particular need to understand MoGA for chemicals which cannot be  
13          subjected to *in vivo* genotoxicity tests (e.g. cosmetics). In this particular  
14          instance some useful additional information on genotoxicity may be provided  
15          by undertaking *in vitro* tests using reconstructed human skin. The  
16          recommended two core genotoxicity tests in Stage 1 are the *in vitro* bacterial  
17          gene mutation test and *in vitro* micronucleus test (MNvit). These  
18          recommended assays provide sufficient information for the genotoxicity  
19          assessment of most chemicals. Information from non-core tests described in  
20          this document may provide useful additional information on *in vitro*  
21          mutagenic hazards on a case-by-case basis. In most instances misleading  
22          negative results are due to inadequate exogenous metabolic activation.  
23          However, in some instances regulatory authorities may require an *in vivo*  
24          genotoxicity test where high, or moderate and prolonged, levels of exposure  
25          are expected (e.g. most human medicines) in order to provide additional  
26          reassurance even when Stage 1 tests have given negative results. If a chemical  
27          is considered on the basis of Stage 1 test results to have *in vitro* mutagenic  
28          potential but has not been tested *in vivo*, the COM considers it prudent to  
29          assume that the chemical may have *in vivo* mutagenic potential.

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1 **Stage 2: *In Vivo* Genotoxicity Tests (Figure 3)**

2 Overview of Strategy

3 63. Stage 2 of the testing strategy involves an assessment of genotoxic activity *in*  
4 *vivo* in somatic tissues and in germ cells (when there is a need for the  
5 assessment of heritable effects and/or information on hazard classification of  
6 mutagens) (see Figure 3). The *in vivo* genotoxicity testing strategy has to be  
7 designed on a case-by-case basis and can be used to address the following  
8 aspects of *in vivo* mutagenicity;

- 9 1) Investigation of mutagenic end point(s) identified in Stage 1,  
10 2) Investigation of genotoxicity in tumour target tissue(s),  
11 3) Investigation of potential for germ cell genotoxicity,  
12 4) Investigation of *in vivo* mutagenicity for chemicals where there is high or  
13 moderate and prolonged exposure.

14 It is thus possible for there to be one or more separate Stage 2 strategies  
15 designed to assess points 1)-4) for a particular test substance. This rationale  
16 leads to different approaches from those advocated by the COM in 2000  
17 where the weight of available evidence suggested that the *in vivo* bone marrow  
18 (or peripheral blood) micronucleus assay or bone marrow clastogenicity assay  
19 in rodents was the preferred first test in almost all cases. The exception was  
20 for direct acting DNA reactive mutagens where a site of contact test was  
21 preferred. There was a preference in the 2000 COM guidance for the rat liver  
22 UDS assay as a second tissue *in vivo* test, which was selected primarily to  
23 provide reassurance of absence of *in vivo* genotoxicity when positive results  
24 had been obtained *in vitro* but negative results were obtained in an *in vivo*  
25 bone marrow micronucleus or chromosomal aberration assay. The selection  
26 of rat liver UDS was based largely on experience in use and the availability of  
27 an OECD guideline (DOH., 2000). A revised *in vivo* Stage 2 strategy based  
28 on the selection of tests to provide information on one or more specific aspects  
29 such as species and/or tissue genotoxicity combined with investigation of  
30 particular genotoxic end points and modes of genotoxic action would not

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1 necessarily lead to the selection of rodent bone marrow micronucleus test as  
2 the first assay or the rat liver UDS assay as a second tissue assay.

3 64. Other factors that should be considered when determining an *in vivo*  
4 genotoxicity testing strategy include whether the testing strategy can be  
5 integrated into other regulatory toxicity tests (such as subacute or subchronic  
6 toxicity studies). Consideration needs to be given to the nature of the  
7 chemical (including physico-chemical properties), the results obtained from *in*  
8 *vitro* genotoxicity tests and the available information on the toxicokinetic and  
9 metabolic profile of the chemical (for example when selecting most  
10 appropriate species, tissue and end point). The routes of exposure in animal  
11 studies should be appropriate to ensure that the substance reaches the target  
12 tissue. Routes unlikely to give rise to significant absorption in the test animal  
13 should therefore be avoided. Unless systemic exposure can be confirmed from  
14 other toxicological studies, or evident toxicity in the target organ is seen,  
15 confirmatory toxicokinetic studies to measure blood or tissue exposure as  
16 appropriate should be undertaken to accompany all *in vivo* genotoxicity  
17 studies to assess the adequacy of any negative results obtained.

18 65. The design of *in vivo* genotoxicity tests should incorporate appropriate  
19 approaches to reduce the number of animals used in such tests. Options for  
20 reduction in animal usage include:

- 21 • Use of one sex only (if supported by metabolism data or other data  
22 indicating equivalence),
- 23 • reduced numbers of sampling times for micronucleus and  
24 chromosomal aberration assays when repeat dosing is performed,
- 25 • integration of micronucleus and comet end points into repeat-dose  
26 toxicity studies,
- 27 • combining micronucleus and comet assays into a single acute test  
28 employing a few administrations of test chemical (Pfuhler et al., 2009,  
29 Bowen et al., 2010, Vasquez, 2010)).

30 It should also be possible to omit the concurrent positive control  
31 administrations in micronucleus and chromosomal aberration tests (but not for

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- 1 the comet assay) where the test facility has appropriate historical positive  
2 control data (Pfuhrer et al., 2009) as long as positive control slides “banked”  
3 from previous treatments and coded in with the experimental slides, are  
4 included to demonstrate proficiency.
- 5 66. The toxic properties of test substances (such as acute toxicity, subchronic  
6 toxicity (including target organ effects), irritancy/corrosivity in contact with  
7 skin or mucous membranes), toxicokinetic and metabolism data will influence  
8 the choice of route of administration and the highest dose level achievable in  
9 *in vivo* mutagenicity tests. Dose selection for *in vivo* genotoxicity testing  
10 requires estimation of the maximum tolerated dose, consideration of tissue-  
11 specific effects and appropriate toxicokinetic data to support tissue exposure to  
12 the substances and/or metabolites.
- 13 67. The approach outlined to Stage 2 in figure 3 takes account of evidence to  
14 suggest that *in vivo* comet and rodent transgenic mutation assays have better  
15 sensitivity and specificity for the identification of rodent carcinogens  
16 compared with the rat liver UDS test, particularly for carcinogens that are  
17 negative in the *in vivo* micronucleus test (Kirkland and Speit, 2008). The  
18 initial *in vivo* genotoxicity testing strategy should therefore involve selection  
19 of one or more of the core Stage 2 tests in rodents; namely, the transgenic gene  
20 mutation tests, micronucleus tests (accompanied by specific assays for  
21 aneuploidy if necessary) or comet DNA damage assays in rodents. It is  
22 acceptable to undertake one *in vivo* genotoxicity test to investigate a specific  
23 mutagenic end point identified from Stage 1 *in vitro* genotoxicity tests. In  
24 some instances there may be a need to investigate more than one end point  
25 before reaching a full conclusion on *in vivo* mutagenic potential.
- 26 68. Stage 2 *in vivo* genotoxicity tests should be undertaken for test substances that  
27 are positive in any of the *in vitro* Stage 1 genotoxicity tests where there is a  
28 need to ascertain whether genotoxic activity can be expressed *in vivo*. There  
29 are many reasons why activity shown *in vitro* may not be observed *in vivo* (for  
30 example, lack of absorption, inability of the active metabolite to reach DNA,  
31 rapid detoxication and elimination). Data from *in vivo* genotoxicity tests are,  
32 therefore, essential before any definite conclusions can be drawn regarding the

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1 potential mutagenic hazard to humans from test substances which have given  
2 positive results in one or more *in vitro* genotoxicity tests. However,  
3 conclusions on mutagenic hazard and MoGA may have to be derived from *in*  
4 *vitro* genotoxicity data for test substances when no *in vivo* genotoxicity testing  
5 is permitted.

6 69. In addition, an *in vivo* genotoxicity test may give positive results for chemical  
7 substances which only act *in vivo*; experience though, has shown that such  
8 chemicals are rare (Tweats et al., 2007b). In some instances positive results  
9 might be obtained from *in vitro* genotoxicity tests that are adapted to evaluate  
10 specific characteristics of the test substance; for example, by the use of  
11 modified or non-standard exogenous metabolising fractions (Muller et al.,  
12 2003b).

13 70. Positive results in any Stage 2 genotoxicity test should be assessed for  
14 evidence of mode of action and for evidence of irrelevant positive responses.  
15 Examples of irrelevant modes of action in micronucleus tests, for instance  
16 include compound induced hypothermia in rodents and compound induced  
17 increases in cell division of bone marrow erythroblasts (Tweats et al., 2007a,  
18 Blakey et al., 2008). If the conclusion is reached that a genotoxic mode(s) of  
19 action occurs then the chemical should be considered as an *in vivo* mutagen.  
20 MoGA data will be important in considering whether a threshold or non-  
21 threshold approach to risk assessment can be used. The COM has published  
22 guidance  
23 (<http://www.iacom.org.uk/guidstate/documents/Thresholdsforinternetfinal.pdf>)  
24 on possible threshold modes of genotoxicity which can include; i) involvement  
25 of non-DNA targets, (e.g. aneugen inhibition of microtubules), ii) the  
26 contribution of protective mechanisms (e.g. repair of DNA adducts formed  
27 from small molecular weight alkylating agents and, iii) overload of  
28 detoxication pathways (e.g. paracetamol).

29 71. Supplementary *in vivo* tests should be undertaken if the results of the initial  
30 core *in vivo* genotoxicity tests give equivocal results or if there is a need to  
31 investigate specific mutagenic endpoints, tumour target organs, or the potential  
32 for heritable effects. This may involve repeating all or aspects of the initial

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1 Stage 2 testing strategy, or performing supplementary investigations (e.g.  
2 mode of action investigations, such as DNA adducts or more specific germ  
3 cell testing) to investigate aspects of the genotoxicity of the test substance  
4 which have not been resolved. There is a need to select the most appropriate  
5 test(s) on a case-by-case basis. All relevant factors, such as results from  
6 previous tests, and available information on toxicokinetics, toxicological  
7 effects and metabolism of the chemical, should be considered.

8 72. One aspect of the approach to testing outlined in Figure 3 is that hazard  
9 characterisation of germ cell genotoxicity can be included in the initial *in vivo*  
10 genotoxicity testing strategy. This is because there are multi tissue *in vivo*  
11 genotoxicity assays (e.g. transgenic mutation assays and comet assay) which  
12 can also be used if a need to evaluate germ cell genotoxicity has been  
13 established although further evaluation of these assays for this purpose is  
14 recommended. Additionally, a small number of germ cell mutation assays  
15 might be valuable on a case-by-case basis to provide information on heritable  
16 mutagenic effects but these would form part of a supplementary *in vivo*  
17 genotoxicity testing strategy, if considered appropriate.

18 73. The COM reaffirms that a chemical considered a positive *in vivo* somatic cell  
19 mutagen should also be considered as a possible germ cell mutagen unless  
20 data can be provided to the contrary. The position held previously, that most  
21 if not all germ cell mutagens are also genotoxic in somatic cells still holds  
22 true. It has been noted that there are some rare examples (e.g. sodium  
23 orthovanadate Attia et al., 2005) where the mouse bone marrow micronucleus  
24 assay does not predict germ cell genotoxicity. However the data on such  
25 compounds are conflicting and it is not known, for example, whether somatic  
26 mutations would have been identified if other test systems (e.g. transgenic  
27 assays) had been used (Ciranni et al., 1995, Witt et al., 2003, Attia et al.,  
28 2005). It is possible these examples may relate to cellular targets in germ cells  
29 that are not present in the bone marrow (e.g. different proteins in chromatin  
30 structure and processes involved in meiosis). However, induction of other  
31 genotoxic effects and in other tissues cannot be excluded. There are also  
32 examples of germ cell mutagens which affect specific stages of gametogenesis

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1 in males (Adler, 2008) and where there are differences between male and  
2 female germ cell genotoxicity (Bishop, 2003).

3 74. It is plausible that other targets during the process of meiotic cell division  
4 may be unique to germ cells but not necessarily identical in both sexes  
5 (Pacchierotti et al., 2007). The COM considers that further research to  
6 understand better the effects of genotoxic substances on mammalian germinal  
7 cells may be informative with regard to genotoxicity testing strategies in the  
8 future. It is noted that some initial results with oocyte *in vitro* and *in vivo*  
9 systems have provided information on germ-cell specific modes of action (Yin  
10 et al., 1998, Ranaldi et al., 2008, Vogt et al., 2008).

#### 11 Discussion of Stage 2 Initial Testing Strategy- General Aspects

12 75. There are many recent publications debating *in vivo* genotoxicity testing  
13 strategies, for example, the German Speaking section of the European  
14 Environmental Mutagen Society recommended a single study using a  
15 combined analysis for micronuclei and comet induction in selected tissues  
16 (Pfuhler et al., 2007) while the WHO/IPCS recommended cytogenetics (bone  
17 marrow) or gene mutation or alternative tests as defined by MoGA, chemical  
18 class and reactivity (with consideration of factors such as bioavailability and  
19 metabolism) (Eastmond et al., 2009). The *in vivo* genotoxicity testing strategy  
20 recommended by the COM acknowledges there can be a variety of reasons for  
21 undertaking *in vivo* genotoxicity tests and it is important to identify clearly the  
22 critical aspects of *in vivo* genotoxicity to be addressed (as set out in the  
23 Overview of Stage 2 strategy set out in paragraph 63 above) which have to be  
24 investigated in order to develop a strategy accordingly, rather than simply  
25 specify preferred first and second tests. There are less data on the  
26 performance of *in vivo* genotoxicity assays for prediction of rodent  
27 carcinogenicity compared to data on the performance of *in vitro* genotoxicity  
28 tests. Transgenic rodent assays (TGR) and the *in vivo* micronucleus assay  
29 have been shown to exhibit significant complementarity regarding prediction  
30 of rodent carcinogenicity, consistent with the assessment of different  
31 mutagenic end points by these two assays (Lambert et al., 2005). TGR was  
32 usually positive for those carcinogens which were positive in *in vitro* gene

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1 mutation tests in bacteria whilst the *in vivo* MN assay had greater predictivity  
2 for carcinogens positive in the *in vitro* metaphase analysis in mammalian cells  
3 (Lambert et al., 2005). Thus MoGA analysis of *in vitro* mutagenic activity is  
4 of considerable importance in helping to develop an initial *in vivo* genotoxicity  
5 testing strategy. The COM recommends that the initial *in vivo* genotoxicity  
6 testing strategy should be based on one or more tests selected from a relatively  
7 limited number of *in vivo* genotoxicity tests that have been specifically  
8 designed to provide the optimum amount of information on *in vivo* mutagenic  
9 potential of the test substance.

#### 10 Discussion of Stage 2-Recommended *In Vivo* Genotoxicity Tests

11 76. Three recommended *in vivo* genotoxicity tests are outlined below and in  
12 Figure 3. Information from one or more of these recommended core tests  
13 should provide sufficient *in vivo* genotoxicity data for most chemicals.

#### 14 *Transgenic Rodent Mutation (TGR) Assay for Gene Mutations*

15 77. There has been a significant increase in the number of studies undertaken with  
16 transgenic rodent mutation (TGR) assays published since the COM guidance  
17 published in 2000. These have been comprehensively reviewed (Lambert et  
18 al., 2005). There are sufficient data to assess the performance of the  
19 Muta<sup>TM</sup> mouse, BigBlue<sup>®</sup> mouse and rat (including use of  $\lambda$  cII transgene),  
20 *LacZ* plasmid mouse, and the *gpt* delta mouse models. The TGR assay can be  
21 used to assess gene mutations in a wide range of rodent tissues (including  
22 germ cells) using all routes of administration (Lambert et al., 2005) and is  
23 particularly valuable when investigating gene mutation as the MoGA. TGR  
24 assays have been reported to produce data that are generally compatible with  
25 the mouse specific locus test for germ line mutagens (Singer et al., 2006a). In  
26 addition TGR assays can be particularly useful for *in vivo* site-of-contact  
27 mutagen assessment (Dean et al., 1999). Guidance on appropriate approaches  
28 to protocol development has been published by the IWGT (Thybaud et al.,  
29 2003). Molecular analysis of induced mutations in transgenic targets can aid  
30 in interpretation of study results (particularly equivocal responses) and also  
31 provide mechanistic information. Further information particularly on non-  
32 carcinogens is required to assess the overall performance of TGR assays

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1 although the available data suggests best positive and negative predictivity  
2 was obtained using results from *in vitro* Salmonella mutagenicity tests and *in*  
3 *vivo* TGR assays (Lambert et al., 2005). There is a need to consider and  
4 validate the optimal protocol when using transgenic mutation tests with tissues  
5 with a slow turnover. The OECD published a Detailed Review Paper (DRP)  
6 on Transgenic Rodent (TGR) Gene Mutation Assays in 2009 and  
7 recommended the development of an OECD guideline (OECD, 2009). A draft  
8 OECD guideline is currently under consideration (OECD, 2010).

9 *Rodent Bone Marrow MN And CA Assays for Clastogenicity and Aneuploidy*

10 78. The *in vivo* bone marrow micronucleus assay is still the most widely used *in*  
11 *vivo* genotoxicity test. Most of the available *in vivo* data on the mutagenicity  
12 of chemicals have been obtained from studies using the bone marrow  
13 micronucleus assay (BMMN) in mice. The bone marrow is readily accessible  
14 to chemicals that are present in the blood and a wide range of structurally  
15 diverse clastogens and aneugens has been detected using these methods. The  
16 BMMN assay detects clastogenicity by measuring micronuclei (MN) formed  
17 from acentric chromosome fragments in young (polychromatic) erythrocytes  
18 in the bone marrow (or reticulocytes of peripheral blood). It may also be used  
19 to identify the induction of numerical aberrations. Micronuclei containing  
20 whole chromosomes (as opposed to fragments) can be identified with  
21 molecular kinetochore or centromeric labelling techniques. It should be noted  
22 that only aneuploidy produced by chromosome loss can be measured in the  
23 BMMN assay. The use of peripheral blood is an alternative approach for both  
24 mice (CSGMT, 1995) and rats (when the youngest fraction of reticulocytes are  
25 sampled) (Wakata et al., 1998, Torous et al., 2000, Suzuki et al., 2005a). High  
26 throughput approaches to the peripheral blood MN assay have been published  
27 (Torous et al., 2000, De Boeck et al., 2005). The rodent micronucleus assay  
28 can be used in the initial *in vivo* genotoxicity strategy for generic testing for *in*  
29 *vivo* mutagenic potential and for assessment of clastogenicity and aneuploidy.  
30 Clastogenicity may be measured by metaphase analysis of chromosomal  
31 aberrations (CA) in bone marrow of rodents as an alternative approach to the  
32 use of the micronucleus assay.

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1 79. Proposals have been published to incorporate micronucleus assays into routine  
2 rodent 28 day subacute toxicity studies which have demonstrated the  
3 feasibility of such an approach,(Kirshna et al., 1998, Hamada et al., 2001,  
4 Madrigal-Bujaidar et al., 2008). The development of a simultaneous liver and  
5 peripheral blood micronucleus assay in young rats has also been reported  
6 (Suzuki et al., 2005b). The evidence from one evaluation of micronucleus  
7 tests conducted on samples from short-term, subchronic and from a few  
8 chronic studies in mice has been published. MN in polychromatic  
9 erythrocytes represent DNA damage occurring in the last 72h, whilst MN in  
10 normochromatic erythrocytes represented average damage during the 30 day  
11 period prior to sampling (Witt et al., 2000).

#### 12 *Rodent Comet Assay for DNA Damage*

13 80. The *in vivo* comet detects a wide spectrum of DNA damage including  
14 repairable DNA damage. An overview of the types of genetic lesions detected  
15 is given above in paragraph 61. The *in vivo* comet assay can detect substances  
16 that induce gene mutations and has produced positive results for nearly 90% of  
17 rodent carcinogens not detected by the rodent bone marrow MN assay  
18 (Kirkland and Speit, 2008). There have been significant developments with  
19 regard to the conduct of the *in vivo* alkaline comet assay since 2000 (Hartmann  
20 et al., 2003, Brendler-Schwaab et al., 2005, Burlinson et al., 2007). This assay  
21 can be used for elucidating positive *in vitro* genotoxicity findings and to  
22 evaluate genotoxicity in target organs of toxicity (Hartmann et al., 2004)  
23 however, it would not be an appropriate follow-up for a substance  
24 demonstrating an aneuploidy mode of action based on *in vitro* findings. There  
25 is now consensus agreement on a protocol for most tissues which would be  
26 consistent with an OECD guideline (Burlinson et al., 2007). The comet assay  
27 can be used in a wide range of species with any tissue including germ cells and  
28 can be applied to site-of-contact tests. In the absence of data indicating  
29 particular tissues of interest (e.g. toxic findings or tissue accumulation seen in  
30 other studies), comet analysis of the stomach/duodenum (to detect site of  
31 contact effects), and liver (to detect genotoxic metabolites) should be studied.  
32 The Committee considers that the *in vivo* comet assay has appropriate

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1 sensitivity to detect chemicals which induce both gene mutations and  
2 clastogenicity. With regard to the assessment of germ cell genotoxicity  
3 measurement of DNA, effects by the comet assay in sperm requires additional  
4 steps for chromatin decondensation. A protocol for standardisation of the  
5 germ cell comet assay has not yet been achieved (Speit et al., 2009). Thus the  
6 *in vivo* comet assay can be used as a core test in the initial *in vivo* genotoxicity  
7 testing strategy to assess DNA damage in multiple tissues in a single study and  
8 it is possible to include the comet assay within standard regulatory toxicity  
9 tests (Rothfuss et al., 2010) or within other *in vivo* genotoxicity tests (Vasquez,  
10 2010) .

#### 11 *Non-Core In Vivo Test: Rat Liver UDS Assay for DNA Damage*

12 81. The rodent liver UDS assay is an established approach for investigating  
13 genotoxic activity in the liver (Kennelly et al., 1993). The endpoint measured  
14 is indicative of DNA damage and subsequent repair in liver cells. The COM  
15 consideration of this assay and published evaluations suggest it gives broadly  
16 similar results to the *in vivo* comet assay with regard to identification of  
17 genotoxicity in the liver (<http://www.iacom.org.uk/statements/UDS.htm>). An  
18 analysis of the prediction of rodent carcinogens not identified by the  
19 micronucleus tests indicated that the comet assay was considerably better than  
20 the rat liver UDS assay at identifying rodent carcinogens (Kirkland and Speit,  
21 2008). The COM's preference is to use the comet assay rather than rodent  
22 liver UDS in order to assess *in vivo* potential for DNA damage.

#### 23 Discussion of Stage 2-Supplementary Tests.

24 82. Supplementary *in vivo* genotoxicity tests need to be considered on a case-by-  
25 case basis taking into account all relevant information. It is considered that for  
26 most chemicals, supplementary *in vivo* genotoxicity data should be  
27 unnecessary but on a case-by-case basis specific aspects of MoGA (e.g. nature  
28 of DNA adducts) and further characterisation of germ cell genotoxicity (e.g.  
29 characterisation of male and/or female germ cell clastogenicity including use  
30 of FISH, and the evaluation of heritable effects) may be required. DNA  
31 adduct studies can provide valuable information on potential genotoxicity as a  
32 follow up for *in vitro* mutagens which have yielded negative results in *in vivo*

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1 genotoxicity assays (Phillips et al., 2000). DNA adduct data (including type of  
2 adduct, frequency, persistence, repair process) should be considered in  
3 conjunction with other relevant data such as dosimetry, toxicity, genotoxicity  
4 and tumour data (Jarabek et al., 2009).

5 83. A brief outline of these additional Stage 2 methods is given in Table 1.  
6 Reference is also made in Table 1 to a number of tests for heritable genotoxic  
7 effects but it is noted that these tests which involve the use of many animals  
8 and demand a high level of expertise are comparatively rarely used. The  
9 COM is aware that there is the possibility that gender differences in germ cell  
10 mutagenesis may exist and this aspect may need to be considered on a case-  
11 by-case basis (Eichenlaub-Ritte et al., 2007).

12

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1 Table 1. Supplementary *in vivo* genotoxicity tests.  
2

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA Adducts				
<sup>32</sup> P-postlabelling	DNA adducts	IWGT	DNA can be extracted. Can be highly sensitive particularly with bulky adducts and if appropriate enrichment technique used.	Interpretation of results can be complex. Involves handling high-activity <sup>32</sup> P.(Phillips et al., 2000)
<b>Covalent binding to DNA</b>  A variety of methods can be used such as those involving radioactive delay or isotopic measurements. (eg. <sup>14</sup> C-) or isotope measurements (eg Accelerator Mass Spectrometry AMS)	DNA Adducts	IWGT	Can be applied to all tissues. Some methods (AMS) are potentially very sensitive and can provide data on DNA binding at levels of exposure similar to low level environmental exposures	Generally radiolabelled compound (very small amounts (nanograms) in this case of AMS). Interpretation of results can be complicated (e.g. by non-specific binding).(Himmelstein et al., 2009;)
<b>Supplementary investigations of germ cell mutagenicity</b>				
Analysis for clastogenicity/aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes		Can provide information on nature of effects in spermatogonia, spermatocytes and/or oocytes of mice or rats	Can provide useful information on MoGA.(Russo, 2000)
Spermatid micronucleus assay	chromosomal aberrations and or lagging chromosomes		Provides information of clastogenic and/or aneugenic effects in spermatocytes.	(Allen et al., 2000)
Dominant lethal assay	Chromosomal/gene mutations	OECD	Provides information on unstable chromosomal changes in gametes that lead to fetal death after fertilization and can determine stage(s) of gametogenesis affected	Little used. needs relatively large numbers of animals(Adler et al., 1994)
Mouse specific locus test	Gene mutations	EPA	Provides information on genetic changes transmitted to the first generation progeny as basis for estimation of induced mutation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on chromosomal changes transmitted to the first generation progeny as basis for estimation of induced translocation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Sperm comet assay	Double strand breaks and/or apurinic sites in sperm head DNA		Provides information on genetic instability in sperm	(Trivedi et al., 2010)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes		Provides information on induction of DNA lesions	(Sotomajor and Segal, 2000)

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1

2 **Summary Stage 2 (*In Vivo* Genotoxicity Testing).**

3 84. The *in vivo* genotoxicity testing strategy has to be designed on a case-by-case  
4 basis and can be used to address the following aspects of *in vivo* mutagenicity;

- 5 1) Investigation of mutagenic end point(s) identified in Stage 1,  
6 2) Investigation of genotoxicity in tumour target tissue(s),  
7 3) Investigation of potential for germ cell genotoxicity,  
8 4) Investigation of *in vivo* mutagenicity for chemicals where there is high or  
9 moderate and prolonged exposure.

10 The recommended *in vivo* genotoxicity test(s) include transgenic rodent  
11 mutation assay, micronucleus assay and comet assay in rodents. In some  
12 instances there may be a need to undertake more than one *in vivo* test to  
13 perform an initial assessment of *in vivo* mutagenic potential (e.g. where  
14 endpoints cannot be assessed in one study and there is a need to investigate  
15 multiple end points before reaching conclusions on *in vivo* mutagenic  
16 potential). Multiple endpoints may be combined in a single study. If positive  
17 results are obtained it is important to consider the evidence for genotoxic  
18 mode of action and check the data for evidence of irrelevant positive results. If  
19 negative results are obtained in the first *in vivo* test then further test(s) would  
20 only be needed if the chemical was clearly positive in a Stage 1 *in vitro*  
21 genotoxicity test and there were also aspects of the genotoxicity that have not  
22 been fully resolved (e.g. for chemicals that affect multiple mutagenic end  
23 points or in the case where an investigation of heritable effects was required).  
24 If equivocal results are obtained, then supplementary testing may be needed.  
25 The supplementary *in vivo* genotoxicity testing strategy should also be devised  
26 on a case-by-case basis. This may involve repeating some aspects of the  
27 recommended *in vivo* genotoxicity tests, or performing supplementary  
28 investigations (e.g. MoGA, such as DNA adducts and/or more detailed  
29 consideration of heritable effects). There is a need to select the most  
30 appropriate assay(s) on a case-by-case basis. All relevant factors such as  
31 results from previous tests, structural alerts and available information on

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1 toxicokinetics and metabolism of the substance, should be considered. In the  
2 absence of appropriate germ cell genotoxicity data, the COM consider it is  
3 reasonable to assume that all somatic cell mutagens have the potential to be  
4 germ cell mutagens.

## 5 **Possible Future Developments**

6 85. The COM is aware that new assays and toxicogenomic approaches are under  
7 development which might be of value within genotoxicity testing. These  
8 include the detection of gene mutations at the endogenous  
9 phosphatidylinositol glycan complementation group A gene (*Pig-A*), a reporter  
10 gene in peripheral red blood cells of mammals (Bryce et al., 2008b, Miura et  
11 al., 2009) and investigation of instability in expanded simple tandem repeats in  
12 male gametes and offspring to evaluate heritable mutations (Singer et al.,  
13 2006b). The development of new high throughput assays for the assessment  
14 of germ line mutations and the quantification of risk from such data may  
15 provide opportunities to protect future generations from mutated DNA  
16 sequences. Developments within the field of toxicogenomics are also likely to  
17 provide new methods for identifying genotoxic mechanisms. The COM have  
18 reviewed data generated in this field several times during 2008 and 2009 up to  
19 the drafting of this guidance statement but currently conclude that the evidence  
20 does not support the routine use of toxicogenomic approaches to genotoxicity  
21 testing (<http://www.iacom.org.uk/papers/index.htm>). A recent workshop held  
22 by the HESI (Health Effects Institute) IVGT (In-Vitro Genetic Toxicity  
23 Testing) Project Committee reviewed 16 assays/technologies which were at  
24 various stages of development (defined as emerging to mature). The  
25 workshop highlighted emerging approaches to genotoxicity testing such as  
26 Enzyme-DNA films and DNA adductome studies which should be considered  
27 as part of the COM's remit to horizon scan for new developments (Lynch et  
28 al., 2010)

29

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1

2 **Annex 1: Sensitivity and Specificity Data Considered by The COM.**

- 3 1. Data for sensitivity (correct identification of rodent carcinogens) and  
 4 specificity (correct prediction of non-carcinogens as assessed in rodent  
 5 carcinogenicity bioassays) have been obtained from a number of publications.  
 6 Information is available for SAR approaches, screening tests and genotoxicity  
 7 assays (both individually and in combinations).  
 8  
 9 2. The figures quoted depend on the carcinogenicity data set used (e.g. Gold  
 10 Carcinogenicity Potency database (<http://potency.berkeley.edu/>), the  
 11 classification of genotoxicity test results (i.e. positive, negative, equivocal  
 12 based on study authors results or subjected to independent peer review) and  
 13 whether equivocal and/or technically compromised (inadequate) test results  
 14 have been included in the analyses. Sensitivity/specificity data for  
 15 genotoxicity tests using genotoxic carcinogens have not been published,  
 16 because this would require considerable work to evaluate the mode of action  
 17 for carcinogenicity for a large number of chemicals. Specificity data for  
 18 identification of chemicals with no *in vivo* genotoxic activity (non-genotoxins)  
 19 have not been published, as there are no published databases for such  
 20 chemicals.  
 21  
 22 3. The Sensitivity and specificity data that have been reviewed by the COM are  
 23 tabulated below (rounded to whole numbers)  
 24

25 Structure Activity Assessments

26

Method	Sensitivity Identification of mutagens or rodent carcinogens	Specificity Identification of non- mutagens or rodent non-carcinogens	Comments/references
DEREK	No data reported	No data reported	Concordance with Ames positive 65% (416 compounds)(Cariello et al., 2002)
TOPKAT	No data reported	No data reported	Concordance with Ames positive 73% (416 compounds (Cariello et al., 2002)
MDL QSAR	81%	76%	3338 compounds tested in bacterial mutagenicity tests (Contrera et al., 2005)
MultiCASE (MC4PC)	71% (bacterial) 63% (mouse lymphoma) 44% (clastogenicity in vitro) 53% (clastogenicity)	88% (bacterial) 74% (mouse lymphoma) 92% (clastogenicity in vitro) 75% (clastogenicity)	1485 compounds, bacterial. 328 compounds for mouse lymphoma. 556 compounds for clastogenicity.(Matthews et al., 2006) 679 compounds(Roithfuss et

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			al., 2006)
Toxtree (version 1.50)	74% (rodent carcinogenicity) 85% (bacterial mutagenicity)	64% (rodent carcinogenicity) 72% (bacterial mutagenicity)	878 chemicals with carcinogenicity data, 698 chemicals with mutagenicity data (Benigni and Bossa, 2008)

1  
2  
3

## Screening Tests

Method	Sensitivity Identification of mutagens	Specificity Identification of non-mutagens	Comments/references
HepG2 (cystatin, p53, Nrf2) luciferase reporter	85% (17/20 ECVAM list) 74% (bacterial mutagenicity) 45% (clastogenicity)	81% (34/42 ECVAM list) 80% (bacterial mutagenicity) 83% (clastogenicity)	62 ECVAM listed chemicals, 192 additional chemicals (Westerink et al., 2010)
Vitotox™ (bacterial SOS reporter assay for mutagenicity) RadarScreen (RAD54 reporter assay in yeast for clastogenicity)	70% bacterial mutagenicity (14/20 ECVAM list) 86% (bacterial mutagenicity).  70% clastogenicity (14/20 ECVAM list), 77% (clastogenicity)	93% (39/42 ECVAM list) 94% (bacterial mutagenicity)  83% clastogenicity (35/42 ECVAM list) 74% (clastogenicity)	62 ECVAM listed chemicals, 192 additional chemicals (Westerink et al., 2009)
GADD45a-GFP	18/20 ECVAM list of mammalian cell mutagens (90%)  63% (regulatory battery of Ames, CA/MNvit, or CA/MLA)  Validation data for 56 compounds requiring metabolic activation  30% 30%	22/23 ECVAM list of mammalian cell non-mutagens (96%)  100% (regulatory battery of Ames, CA/MNT or CA/MLA)  97% 88%	(Birrell et al., 2010)  75 compounds studied (Hastwell et al., 2006)  (Jagger et al., 2008)  57 <i>in vitro</i> genotoxins 50 chemicals with rodent carcinogenicity data. (Olaharski et al., 2009)

4  
5

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1 Genotoxicity Tests (*in vitro*)

2

Method	Sensitivity	Specificity	Comments/references
Ames	59%	74%	541 chemicals (Kirkland et al., 2005a)
Ames	52%	72%	3711 chemicals including tests with <i>Salmonella</i> and <i>Escherichia</i> (Matthews et al., 2006)
Micronucleus ( <i>in vitro</i> )	79%	31%	89 chemicals (Kirkland et al., 2005a)
Micronucleus ( <i>in vitro</i> )	88%	23%	182 chemicals (Matthews et al., 2006)
Chromosomal aberrations ( <i>in vitro</i> )	66%	45%	352 chemicals (Kirkland et al., 2005a)
Chromosomal aberrations ( <i>in vitro</i> )	55%	63%	1391 chemicals (Matthews et al., 2006)
Mouse lymphoma assay	73%	39%	245 chemicals (Kirkland et al., 2005a)
Mouse lymphoma assay	71%	44%	827 chemicals (Kirkland et al., 2005a, Matthews et al., 2006)
Ames + Micronucleus* combined	94%	12%	372 chemicals. Positive results in at least one test. (Kirkland et al., 2005a)
Ames + mouse lymphoma* combined	89%	32%	436 chemicals (Kirkland et al., 2005a)
Ames+ mouse lymphoma +Chromosomal aberrations combined	84%	23%	202 chemicals (Kirkland et al., 2005a)
Ames + mouse lymphoma + micronucleus* combined	91%	5%	54 chemicals (Kirkland et al., 2005a)

3 \*Positive results in at least one test

4

5

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1 Further data on combinations of genotoxicity tests presented to COM at the 17 June  
 2 2010 meeting <http://www.iacom.org.uk/papers/documents/MUT2010-08Slides.pdf>  
 3 and subsequently up-dated in light of re-evaluation of NTP MLA results (Schisler et  
 4 al., 2010)

5 1.

Chemicals evaluated	Sensitivity Ames +MN	Sensitivity Ames +MN +MLA	Comments
557 rodent carcinogens	73% (409/557). Remainder were negative, negative but technically compromised, weak, equivocal or inconclusive/insufficient detail.	75% (420/557) Of the additional 11 carcinogens identified by MLA, 10 were not tested in MN.	No convincing evidence any rodent carcinogens would be 'missed' by Ames + MN.
409 <i>in vivo</i> genotoxins	(78%) 317/409 Remainder were negative, but technically compromised, weak, equivocal or inconclusive/insufficient detail.	(79%) 323/409 4/6 of the additional <i>in vivo</i> genotoxins detected by MLA had not been tested in either MN or CA.	No convincing evidence that any <i>in vivo</i> genotoxins would be 'missed' by Ames + MN.

6

7

8 \*or chromosomal aberration data where no micronucleus test was available.

9 Abbreviations MN= micronucleus test, MLA = mouse lymphoma assay, CA=

10 chromosomal aberration assay

11

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- 1 **Annex 2. Tabulation of Genotoxicity Tests (in Stages 1 and 2) and**
- 2 **Mutagenic/Genotoxicity End Points Detected.**

Genotoxicity test	Mutagenic/genotoxicity end point detected	Comments
Ames	Gene mutation	Responds to wide range of DNA reactive mutagens when full set of <i>S. typhimurium</i> tester strains and <i>E. coli</i> WP2 with appropriate exogenous metabolic activation used.
Micronucleus test	Clastogenicity, aneuploidy	Centromere or kinetochore stains, with pancentromeric or chromosome specific centromeric probes using fluorescence in situ hybridisation (FISH) is required for aneuploidy
Chromosomal aberrations	Clastogenicity, aneuploidy	Indications of aneuploidy from induction of polyploidy or increased mitotic index, but the use of chromosome specific centromeric probes fluorescence in situ hybridisation (FISH) required to assess the potential for aneuploidy. Very similar assay performance compared with micronucleus test
Mouse Lymphoma Assay	Gene mutation, clastogenicity	Distribution of large and small colony mutants can give information on induction of gene mutations versus clastogenicity. No convincing evidence that MLA can detect aneuploidy consistently.
Comet assay	DNA strand breaks and alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action.
Rodent transgenic mutation assay	Gene mutations	Valuable for the investigation of gene mutation in a wide range of tissues including germ cells and particularly to confirm gene mutation as a mode of action.
Rodent Bone Marrow/peripheral blood micronucleus assay	Clastogenicity	A wide range of structurally diverse clastogens have been detected. Can also be used to investigate aneuploidy by use of centromere or kinetochore probes. .
Rodent comet assay	DNA strand breaks, alkali labile sites	Valuable for detection of DNA damage in a wide range of tissues but gives no information modes of mutagenic action.
Rodent Liver UDS	Unscheduled DNA synthesis	endpoint measured is indicative of DNA damage and subsequent repair in liver cells. Broadly similar response compared with comet assay.

3

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1 **Annex 3:**

2  
3 **Rationale for Selection of Ames Test and *In Vitro* Micronucleus Assay as The**  
4 **Two Principle *In Vitro* Assays.(Kirkland Et Al., 2010)**

5  
6 An evaluation of the use of *in vitro* genotoxicity tests to predict rodent carcinogens  
7 (557 chemicals evaluated) and *in vivo* genotoxins (405 chemicals evaluated) was  
8 prepared and updated for the COM meeting in June 2010  
9 (<http://www.iacom.org.uk/papers/index.htm> MUT/2010/08). A two-test battery  
10 consisting of the Ames test plus *in vitro* micronucleus tests correctly identified 73%  
11 of rodent carcinogens. This is lower than in the published sensitivity analysis of  
12 Kirkland et al (Kirkland et al., 2005a), because the current analysis the *in vitro*  
13 chromosomal aberration test was accepted as a surrogate for the *in vitro* micronucleus  
14 test where no data existed for the latter, as the concordance between the 2 tests for  
15 detection of clastogens is so high. Thus, the denominator used in the calculation of  
16 sensitivity for the current 2-test battery, by taking either *in vitro* micronucleus or *in*  
17 *vitro* chromosomal aberration results, is correspondingly larger than in the Kirkland et  
18 al paper (Kirkland et al., 2005a) and lower sensitivity is reported. By adding the MLA  
19 as a third *in vitro* test, the sensitivity increased marginally to 75%, but of the  
20 additional 11 carcinogens, 10 had not been tested in the *in vitro* micronucleus test and  
21 so it is not known whether they would also have been positive in the *in vitro*  
22 micronucleus as well as in the MLA.

23  
24 A two-test battery of an Ames test and the *in vitro* micronucleus tests correctly  
25 detected 78% of *in vivo* genotoxins. By adding the MLA as a third test the sensitivity  
26 increased marginally to 80%, but of the additional 6 *in vivo* genotoxins, 4 had not  
27 been tested in the *in vitro* micronucleus test and so it is not known whether they  
28 would also have been positive in the *in vitro* micronucleus as well as in the MLA.  
29 From both rodent carcinogen and *in vivo* genotoxin databases there were only four  
30 chemicals for which there was some evidence that the MLA may be more sensitive  
31 than the *in vitro* micronucleus. However, the data are not convincing for the  
32 following reasons:

- 33
- 34 1. Toluene was reported positive in the NTP MLA study, but has subsequently been  
35 re-evaluated as equivocal in the analysis of (Schisler et al., 2010), and was not found  
36 positive in a rigorous MLA conducted to higher concentrations and >80% toxicity  
37 (Kirkland and Fowler, 2010).
  - 38 2. Benzyl acetate was reported positive in the NTP MLA study, and subsequently re-  
39 evaluated as positive by Schisler et al (2010), but an expert panel review (Mitchell et  
40 al, 1997) identified this chemical as "untestable" in the MLA because it reacts with  
41 the plastic of the culture vessels and may thus produce artefacts.
  - 42 3. Morphine was negative in a non-standard *in vitro* micronucleus test in which mouse  
43 splenocytes were treated only for 21 hr in the absence of metabolic activation. It is  
44 possible that morphine may induce micronuclei when tested at higher concentrations  
45 over shorter periods in the absence and presence of metabolic activation in a standard  
46 assay.
  - 47 4. Thiabendazole is an aneugen which, typically, has a very steep dose response. It  
48 has been found positive for induction of micronuclei *in vitro* in several papers, but is

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1 reported equivocal or negative in other papers, possibly because optimum  
2 concentration spacing, treatment and sampling times were not used.

3  
4 Whilst re-testing of these four chemicals, and of several others for which neither in  
5 vitro micronucleus or chromosomal aberration data exist (Kirkland et al., 2010), could  
6 provide additional reassurance, the Committee concluded that, based on the large  
7 amount of available data, there is no convincing evidence that any rodent carcinogen  
8 or *in vivo* genotoxin would be “missed” by using an *in vitro* genotoxicity test battery  
9 consisting of Ames test and *in vitro* micronucleus test. Summary analyses of  
10 sensitivity for the combination of Ames and micronucleus tests is provided in Annex  
11 1.

12  
13 The revised strategy of two tests (Ames and MNvit) allows for the efficient  
14 identification of all mutagenic end points with an optimal low level of misleading  
15 positive results.

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## 1 GLOSSARY

2  
3 A glossary of terms used in COT reports is available which provides useful information on  
4 may of the terms cited in this Com guidance document.

5 <http://cot.food.gov.uk/moreinfo/cotglossary> A glossary of additional terms used in this  
6 guidance document is given below.

### 7 8 9 **Additional terms**

10  
11 **DNA Strand Breakage;** A break in double-stranded DNA in which one or both of  
12 the two strands have been cleaved; both strands have not separated from each other.

13  
14 **DNA Strand Break Assay (Comet assay):** Alkaline treatment converts certain types  
15 of DNA lesions into strand breaks that can be detected by the alkaline elution  
16 technique or by measuring migration rate through a filter, or by the single gel  
17 electrophoresis or Comet assay in which cells embedded in a thin layer of gel on a  
18 microscope slides are subjected to electric current causing shorter pieces of DNA to  
19 migrate out of the nucleus into a Comet tail. The extent of DNA migration is  
20 measured visually under the microscope on stained cells.

21  
22  
23 **Erythrocyte:** red blood cell; corpuscle; one of the formed cells in peripheral blood.  
24 Normally, in humans, the mature form is a non-nucleated, yellowish, biconcave disk,  
25 containing haemoglobin and transporting oxygen.

26 Normochromic erythrocyte; one of normal colour with a normal concentration of  
27 haemoglobin. Polychromatic erythrocyte; one that, on staining, shows shades of blue  
28 combined with tinges of pink indicative of an immature erythrocyte.

29  
30 **Eukaryotes;** A class of organisms, which in contrast to prokaryotes (e.g. bacteria),  
31 comprise cells which have a nucleus in which DNA is organised into characteristic  
32 sets of chromosomes. This includes all plants and fungi except the blue-green algae  
33 and all animals.

34  
35 **Fluorescent in situ hybridization (FISH)** A technique in which a chemically  
36 modified DNA (or RNA) probe is hybridized with target DNA, usually present as a  
37 chromosome preparation on a microscopic slide. The chemical modification can be  
38 visualized using a fluorescent microscope either directly when the modification  
39 involves use of a fluorescent dye or indirectly with the use of a fluorescently labelled  
40 affinity reagent (e.g. antibody or avidin). Depending upon the type of probe used, this  
41 approach can be used to precisely map genes to a specific region of a chromosome in  
42 a prepared karyotype, enumerate chromosomes, or detect chromosomal deletions,  
43 translocations, or gene amplifications in cancer cells.

44 **Gametogenesis** is a process by which diploid or haploid precursor cells undergo cell  
45 division and differentiation to form mature haploid gametes. Depending on the  
46 biological life cycle of the organism, gametogenesis occurs by meiotic division of  
47 diploid gametocytes into various gametes or by mitotic division of haploid  
48 gametogenous cells.

49

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1 **Heritable translocation test** A test that detects heritable structural chromosome  
2 changes (i.e. translocations) in mammalian germ cells as recovered in first-generation  
3 progeny.

4  
5 **Historical negative control data**; In the context of the COM guidance on  
6 genotoxicity testing, this term refers to information on the background genotoxicity or  
7 mutagenicity data for a particular assay from a particular laboratory. Historical  
8 control data should be reported as the mean and confidence intervals for the  
9 genotoxicity or mutagenicity indices investigated.

10 **Kinetochores** is the protein structure which is present on chromosomes where the  
11 spindle fibers attach during division to pull the chromosomes apart. The kinetochore  
12 forms in eukaryotes and assembles on the centromere and links the chromosome to  
13 microtubule polymers from the mitotic spindle during mitosis and meiosis. The  
14 kinetochore contains two regions: an inner kinetochore, which is tightly associated  
15 with the centromere DNA; and an outer kinetochore, which interacts with  
16 microtubules.

17 **Kinetochores staining** An immunochemical technique used to detect the presence of  
18 centromeric kinetochore proteins in micronuclei and to identify the origin of  
19 micronuclei. In all but a few cases, the presence of kinetochore in a micronucleus  
20 indicates that it was formed by loss of an entire chromosome, whereas a micronucleus  
21 that lacks a kinetochore originated from an acentric chromosome fragment.

22  
23 ***In silico***; Computer based simulations of genotoxicity test systems that embody  
24 important aspects (e.g. structural features associated with mutagenicity). The  
25 approach allows prediction of mutagenic activity in specified genotoxicity test  
26 systems (e.g. bacterial mutagenicity).

27  
28 **Maximum Tolerated Dose**; The highest dose of a substance that can be given  
29 without causing serious weight loss (>10%) or other signs of toxicity.

30  
31 **Mode of Genotoxic Action (MoGA)**: The mode of action of genotoxicant refers to the  
32 underlying events involved in the process whereby the chemical induces genotoxic  
33 effects. In order for a specific mode of action to be supported there needs to be  
34 evidence from robust mechanistic data to establish a biologically plausible  
35 explanation. Mode of genotoxic action should be distinguished from the term  
36 mechanism of action. The latter relates to having sufficient understanding of the  
37 molecular basis of the chemical genotoxicity to establish causality. Thus mechanism  
38 of action is at the other end of a continuum from little or no evidence of mode of  
39 genotoxic action to scientific proof of mechanism of action.

40  
41 **Polyploidy**: Numerical deviation of the modal number of chromosomes in a cell,  
42 with approximately whole multiples of the haploid number. Endoreduplication is a  
43 morphological form of polyploidy in which chromosome pairs are associated at  
44 metaphase as diplochromosomes.

45  
46 **Screening test**; High-Throughput procedures designed to provide rapid information  
47 on toxicological end points for a large number of compounds.

This is a draft paper for consultation. All comments should be sent to [Sue.Kennedy@hpa.org.uk](mailto:Sue.Kennedy@hpa.org.uk) as early as possible and by 14 February 2011 at the latest.

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**Prokaryotes:** The simplest living organisms namely viruses, bacteria and some blue green algae. The genetic material in bacteria is arranged into one chromosomal complex consisting of a single circular molecule of DNA (or RNA in some viruses). They lack an organised nucleus. Mitosis and meiosis do not occur, although nucleotide polymerisation replication takes place and division and multiplication follow.

**Recombination:** Breakage of DNA structure with balanced or unbalanced rejoining of DNA

**S9:** metabolic activation system comprising of the post-mitochondrial supernatant (S9) from the homogenised livers of rats treated with P450 dependent drug-metabolizing enzyme inducers such as Arochlor 1254 or phenobarbitone/ $\beta$ -naphthoflavone. S9 is combined with a mix of co-factors which optimize the activity of the mixed function oxidases and form a NADPH generating system.

**Sensitivity;** In the context of the COM guidance on a strategy for genotoxicity testing, the correct identification of rodent carcinogens or *in vivo* genotoxins using genotoxicity (mutagenicity) assays based on a defined set of carcinogenicity data (e.g Gold Carcinogenicity Potency database)

**Specificity;** In the context of the COM guidance on a strategy for genotoxicity testing, the correct prediction of non-carcinogens as assessed in rodent carcinogenicity bioassays using genotoxicity (mutagenicity) assays based on a defined set of carcinogenicity data (e.g Gold Carcinogenicity Potency database).

**Specific locus test** A technique used to detect recessive induced mutations in diploid organisms; a strain that carries several known recessive mutants in a homozygous condition is crossed with a non mutant strain that has been treated to induce mutations in its germ cells; induced recessive mutations allelic with those of the test strain will be expressed in the progeny.

**Spindle apparatus;** In cell biology, the spindle apparatus is the structure that separates the chromosomes into the daughter cells during cell division. It is part of the cytoskeleton in eukaryotic cells. It is also referred to as the mitotic spindle during mitosis and the meiotic spindle during meiosis.

**Topoisomerases** catalyze and guide the unknotting of DNA by creating transient breaks in the DNA using a conserved tyrosine as the catalytic residue. In so-called circular DNA, in which double helical DNA is bent around and joined in a circle, the two strands are topologically linked, or knotted. Topoisomerase I solves the problem caused by tension generated by winding/unwinding of DNA. It wraps around DNA and makes a cut permitting the helix to spin. Once DNA is relaxed, topoisomerase reconnects broken strands

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- 1 **Weight of Evidence** A quantitative ranking of evidence, or the qualitative appraisal
- 2 of many different forms of evidence (e.g toxicological or genotoxicity data) to arrive
- 3 at a conclusion regarding potential hazard (such as mutagenicity).
- 4