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

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

Executive Summary

The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agencies with advice on the most suitable approaches to testing chemical substances for genotoxicity. The COM published guidance in 1981, 1989 and again in 2000. This document reports on the COM views regarding the most appropriate strategy for genotoxicity testing reached in 2011.

The COM recommends a staged approach to testing:

Stage 0 consists of preliminary considerations which include physico-chemical properties of the test chemical substance, Structure Activity Relationships (SAR), and information from screening tests. However data from SAR and screening tests should not overrule test data from adequately designed and conducted genotoxicity tests.

Stage 1 consists of *in vitro* genotoxicity tests. The COM recommends a core-test battery of the Ames test combined with the *in vitro* **micronucleus test. This combination** provides adequate information on three types ~~levels~~ of genetic damage for which data are required (namely, gene mutation, chromosomal damage and aneuploidy) and gives appropriate sensitivity to detect chemical mutagens ~~and carcinogens~~. There is no need to independently replicate adequately designed and conducted core *in vitro* tests which are either clearly negative or clearly positive. The strategy document also considers the value which can be attributed to a number of non-core *in vitro* tests

Stage 2 consists of *in vivo* genotoxicity tests. A case-by case strategy should be developed to answer a number of specific queries;

1) Investigation of mutagenic end point(s) identified in Stage 1,

2) Investigation of genotoxicity in tumour target tissue(s),

3) Investigation of potential for germ cell genotoxicity,

4) Investigation of *in vivo* mutagenicity for chemicals where there is high or moderate and prolonged exposure

5) Investigation of genotoxicity in site of contact tissues.

The core tests in Stage 2 are the rodent micronucleus/chromosome aberration assays for aneuploidy and clastogenicity~~ and aneuploidy~~, the Transgenic rodent gene mutation assay and the rodent C~~c~~omet assay for DNA damage. The strategy document also considers the value which can be attributed to a number of non-core *in vivo* tests. In most instances information from core *in vivo* tests is sufficient to evaluate the *in-vivo* mutagenicity of chemical substances A supplementary *in vivo* test strategy can provide additional information on a case-by-case basis to investigate aspects such as further characterisation of germ cell genotoxicity and DNA adduct data which can inform on the mode of action of carcinogenic chemicals~~genotoxic carcinogens~~.

I. Preface

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

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

3. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to a body ~~n 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. This guidance focuses on testing strategies for

chemical substances for which there are no available genotoxicity data. A separate document providing guidance on a strategy for the mutagenic hazard assessment of chemicals with limited or inadequate genotoxicity data is in preparation. (link). 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 or material including any additive necessary to preserve its stability and any impurity deriving from the process used (see Glossary). (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/sou1765797849217666006088 2). The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with potential 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 the mutagenic hazard of chemicals.

Comment [.1]: To be finalised - awaiting decision on title of other document

Comment [.2]: TO glossary including enlarged definition

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)~~(DOH., 1989)~~. These provided guidance to the relevant government departments and agencies on best practice for testing at those 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)~~(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 the use of live animals where practical and where validated alternative methods are available. It is recognised that, as with the earlier published COM guidance, it may be some time before this strategy is reflected in guidelines used by UK regulatory authorities. ~~Specific guidance is now also given for applying the COM testing strategy to the~~

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~~mutagenicity assessment of chemical substances which have existing, but in many cases inadequate or incomplete, genotoxicity data.~~

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

II. Introduction

6. The COM last published guidance on a strategy for the testing of chemicals for mutagenic potential in 2000 (DOH, 2000)~~(DOH., 2000)~~. The rationale developed by COM in 2000, particularly in relation to the testing of all potential mutagenic endpoints, has also been adopted by the International Workshops on Genotoxicity Testing (IWGT) (Muller et al., 2003b)~~(Müller et al., 2003b)~~. Since 2000 there has been development of new approaches to identifying genotoxic hazards *in vitro* including new approaches to identify misleading positive results and evaluate target organ genotoxicity *in vivo*. There is also a need to develop a testing strategy which can encompass chemicals such as cosmetics where no animal tests are permitted under EU law. It is the objective of this paper to set out a scientifically valid testing strategy comprising those methods which the COM believe to be the most informative with regards to the detection of genotoxic hazard and (when possible) are well validated. There is no discussion of methods which experience has shown to have no place in the recommended genotoxicity testing strategy. Details of methodologies are not given since they are provided in the Organisation for Economic Cooperation and Development

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(OECD) test guidelines, the EU Test Methods Regulation (EC 440/2008) 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) guidance.

7. The genome can be damaged in a variety of ways either spontaneously or from exposure to genotoxic agents. ~~The COM evaluates the results of the available tests on a particular substance and provides advice on mutagenic hazard (i.e. mutagenic potential).~~ The term “mutagenic” refers to the ability of a substance to induce a permanent change in the amount or structure of the genetic material of an organism, which may result in a heritable change in the characteristics of the organism. Chemicals inducing mutations are referred to as mutagens (they are mutagenic). These alterations may involve individual genes, blocks of genes, or whole chromosomes. Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of larger changes, including deletions and rearrangements of DNA. The potential to induce mutation is measured in test systems that detect a broader range of genetic changes than simply mutation – they measure genotoxicity.

Comment [.3]: GUM states - the term ‘gene mutation’ should be used instead of ‘mutation’ . Also use ‘numerical chromosome mutation’ instead of damage. WP DISCUSSED THIS AND REJECTED.

8. Genotoxicity refers to interaction with or damage to DNA and/or other cellular components regulating the fidelity of the genome. Cells have the capacity to protect themselves from such potentially lethal or mutagenic genotoxic effects by many repair processes and therefore many genotoxic events do not become evident as mutations. However, the capacity to damage the genome (genotoxicity) is an indicator of potential mutagenicity. Thus, some methods that measure genotoxicity may not provide direct evidence of inherited mutation.

~~Genotoxic (or genotoxicity) refers to chemicals that interact with or damage the DNA and/or the cellular apparatus which regulates the fidelity of the genome, e.g. the spindle apparatus, and enzymes such as the topoisomerases. It is a broad term that, as well as mutation, includes structural chromosomal damage (clastogenicity), numerical chromosomal damage (aneuploidy, polyploidy) damage to DNA or the production of DNA adducts, by the chemical itself or its metabolites. Genotoxic effects also include DNA strand~~

~~breakage, unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE) and mitotic recombination in yeast. However the detection of such effects does not in itself provide direct evidence of inherited mutations. The term "genotoxic carcinogen" as used by the COM described those chemicals that have been demonstrated to be carcinogenic in humans and/or animals and are considered to be *in vivo* mutagens. Mutagenicity is accepted as a key event in carcinogenicity.~~

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

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

III Significance of Chemical-Induced Mutation for Human Health

11. A mutation in the germ cells of sexually-reproducing organisms may be transmitted to the offspring, whereas a mutation that occurs in somatic cells

may be transferred only to descendant daughter cells. Mutagenic chemicals may present a hazard to health since exposure to a mutagen carries the risk of inducing germ-line mutations, with the possibility of inherited disorders, and the risk of somatic mutations including those leading to cancer.

12. A separate ~~guidance~~ statement discussing ~~on~~ the significance of chemical-induced mutation to human health is in preparation (link?)

IV. General Principles of Testing Strategy

13. The COM recommends a two-stage genotoxicity testing strategy (Stages 1 and 2) for the detection of the mutagenic hazard of chemicals which can be supported by appropriate preliminary screening tests and/or *in silico* data (Stage 0). An overview of the complete strategy is provided in Figure 1. Initial testing for mutagenic potential in Stage 1 is based upon two core *in vitro* tests that are chosen to provide information on gene mutation, clastogenicity and aneuploidy, with case-by-case additional testing and investigation depending on the results of these initial genotoxicity tests. All *in vitro* tests should be designed to provide the best chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system (S9 - see glossary); (b) the ability of the compound or its metabolite(s) to reach the target DNA and/or targets such as the cell division apparatus, and; (c) the ability of the genetic test system to detect the given type of genotoxic event. Where international guidance is available, the assays should be carried out to conform to those~~es~~e internationally recognised protocols e.g. as published by the OECD, the IWGT and in the EU test methods Regulation (EC 440/2008). The same approach to testing can be used for chemical substances where *in vivo* genotoxicity testing is not permitted (e.g. cosmetics). ~~A case-by-case testing strategy should be developed for substances which have existing but possibly inadequate and/or incomplete genotoxicity data.~~ Investigations regarding mode of genotoxic action are important to derive conclusions on biological significance of genotoxicity test results and to inform on the strategy for *in vivo* tests and are particularly important for those chemicals where no *in vivo* genotoxicity testing is permitted.

14. For most chemicals, ~~negative~~ results from the two Stage 1 core tests should be sufficient to reach a conclusion on the presence or absence of mutagenic

potential. However, in some instances, even when Stage 1 tests are negative, regulatory authorities may require consideration of the need for *in vivo* Stage 2 testing particularly where exposure is considered to be high, or moderate and prolonged (e.g. most human medicines). Guidance on the level of exposure which equates to high, moderate or prolonged is beyond the scope of COM guidance. ~~However, if exposures exceed the Threshold of Toxicological Concern (TTC), which has been used as a risk assessment tool predominantly in the food and pharmaceutical sectors, this may be useful to identify priorities for further testing on a case by case basis (Munro et al., 2008).~~

Comment [.4]: SFTG and EEMS thought the ref to TTC here was confusing – it should be used to determine when testing is required.

15. Stage 2 consists of a number of *in vivo* tests designed to investigate whether *in vitro* genotoxic activity including specific mutagenic end points identified by *in vitro* tests can be expressed in the whole animal. This may also include assays for specific target organs (e.g. rodent tumour organs) or in germ cells. Few chemicals are active only *in vivo* and in such cases this may be due to a number of factors such as metabolic differences, the influence of gut flora, higher exposures *in vivo* compared to *in vitro* and pharmacological effects (e.g. folate depletion or receptor kinase inhibition) (Tweats et al., 2007b).

There is currently no single *in vivo* test which can assay all three types ~~levels~~ of genetic damage (Thybaud et al., 2007) and thus a strategy for Stage 2 has to be designed based on the nature of genotoxic effects identified in Stage 1 and the possibility that genotoxic activity will only be expressed *in vivo* as discussed above. However consideration should be given to the possibility of evaluating different genotoxicity endpoints in a single set of test animals.

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16. There should be a clear strategy for planning tests within each stage and for progressing to Stage 2. Clear statements can be made regarding the initial *in vitro* tests to be used in Stage 1 as these methods have been well studied, whereas the strategy for Stage 2 is more complex and, if not a specific regulatory requirement, needs to be developed on a case-by-case basis.

. ~~Few chemicals are active only *in vivo* and in such cases this may be due to a number of factors such as metabolic differences, the influence of gut flora,~~

higher exposures *in vivo* compared to *in vitro* and pharmacological effects (e.g. folate depletion or receptor kinase inhibition) (Tweats et al., 2007b).

17. Under the strategy recommended by COM, the use of animals in mutagenicity testing is primarily required when it is necessary to investigate whether genotoxic activity detected in Stage 1 *in vitro* is reproduced *in vivo,* to study target organ genotoxicity (for example involvement of genotoxicity in rodent tumours (Kirkland et al., 2007c)) and to evaluate the potential for heritable mutagenic effects. The *Iin vivo* genotoxicity testing strategy may also be required by regulatory authorities where there is a likelihood of high, or moderate and prolonged human exposure. Genotoxicity testing using animals should be carried out when there is no suitable alternative, and the minimum number of animals should be used, consistent with obtaining valid results. If feasible, studies can be conducted as an adjunct to single or repeat dose toxicity studies. The COM supports current and future developments to replace, refine or reduce the need for *in vivo* genotoxicity testing.

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VI Genotoxicity Testing Strategy

Comment [.5]: Section for strategy for chemicals with existing data has been removed

189. 21. The COM guidance provides a strategy for testing all chemical substances where no genotoxicity data are available. including those which have existing (and often limited or inadequate) genotoxicity data. Test substances may also contain impurities at varying levels which may also exhibit genotoxic activity. Separate guidance on the genotoxicity assessment of impurities has been identified as a priority project during the COM horizon scanning exercise in 2010 (see minutes of COM meeting of October 2010 http://www.iacom.org.uk/meetings/index.htm), and is currently the subject of an ICH expert working group (M7 –link). The strategy recommended in the following sections is concerned with testing for genotoxic activity of chemical substances and not with mixtures of chemicals. Since the publication of the COM guidance in 2000, assessments of the performance of SAR approaches, screening tests and genotoxicity assays (both individually and in combinations) regarding the prediction of rodent carcinogenicity have been published (Kirkland et al., 2005a, Mathews et al., 2006, Matthews et al., 2006, Kirkland and Speit, 2008)(Kirkland et al., 2005a, Matthews et al., 2006, Matthews et al., 2006, Kirkland and Speit, 2008). Reference to these

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publications can provide an insight into the performance of the genotoxicity assays specifically in relation to the particular data sets analysed and the end points considered predominantly rodent carcinogenicity but also *in vivo* genotoxicity (Kirkland et al., 2011)(Relevant sensitivity and specificity data and assay performance assessments have been summarised in Annex 1 and are discussed further in Annex 3 for information and are cited where appropriate in the text below. Overall the older available data suggest that mammalian cell assays did not perform well at discriminating between rodent carcinogens and non-carcinogens. However, recent data suggest that mammalian cell tests conducted and interpreted according to current recommendations are more robust.

Stage 0: Preliminary Considerations Prior to Genotoxicity Testing

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

Physico-chemical and Toxicological Properties

20. The physico-chemical properties of the test substance (for example, pKa, partition coefficient, solubility, volatility and stability in, and potential reactions with, solvents/vehicles) and its purity can affect the ease of conduct and results of *in vitro* tests. For example, the tolerance of cells to acidic chemicals can be enhanced by neutralisation but this may affect the inherent reactivity of substances to DNA (Hiramoto et al., 1997). Alternatively, low solubility may limit the feasibility of undertaking some or all of the *in vitro* mutagenicity tests recommended in this strategy. The potential for auto-oxidation of the test chemical in the culture medium can also affect the outcome of *in vitro* genotoxicity tests (Long et al., 2007). It is noteworthy that the toxic properties of test substances, such as target organ effects, or irritancy/corrosivity in contact with skin or mucous membranes and their toxicokinetics and metabolism will influence the choice of route of administration and the highest dose level achievable in Stage 2 *in vivo* mutagenicity tests.

Structure Activity Relationships

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Comment [.7]: This section ended up being quite long and several reviewers suggested a table for clarity. Or moving some of para 24 and 25 to an annex

21. Whether the test substance would be expected to have mutagenic potential may ~~can~~ be assessed from its chemical structure, which may provide structural alerts for mutagenicity. A composite model structure was originally devised by Ashby and Tennant in 1991 indicating substituent chemical groups or moieties associated with DNA-reactivity (Ashby and Paton, 1993). A number of freely available and commercial systems to investigate structure activity relationships (SAR) for mutagenicity have been developed and evaluated since 2000 (Zeiger et al., 1996, Cariello et al., 2002, Contrera et al., 2005, Snyder and Smith, 2005, Benigni et al., 2007, Benigni and Bossa, 2008). Further information on various models is provided in Annex 1. The OECD (OECD, 2004)~~(OECD., 2004)~~ and the European Commission (Joint Research Centre) have published principles for the validation of (Q)SAR ((Quantitative) Structure Activity Relationships) (Worth et al., 2005, Benigni and Bossa, 2008). (Q)SAR assessment of the in *vitro* mutagenicity in bacteria has been attained by two types of approach; statistical analyses of structure and mutagenic activity and/or (Q)SAR models using programmed rules for prediction of mutagenic activity based on the available knowledge and expert judgement.

22. Such (Q)SAR systems can be useful when a large number of chemicals require assessment and prioritisation for genotoxicity testing or in instances where a rapid assessment of a chemical is required and there are no genotoxicity test data available. Each (Q)SAR system has a defined domain of applicability which is determined by the structural/descriptor factors, modes/mechanism of mutagenicity, and metabolic aspects included within the system. In addition *in silico* approaches can aid in the interpretation of Stage 1 *in vitro* genotoxicity test results (Dearfield et al., 2010). The available systems perform well for prediction of bacterial mutagenicity (i.e. for chemical structures within the domain of applicability of the model under consideration) (see Annex 1). However, lower sensitivities and specificities have been reported for a number of systems when used for prediction of results from *in vitro* cytogenetics or the mouse lymphoma assay (e.g. using MCASE and MDL-QSAR) (Contrera et al., 2008). One factor in the lower predictive capability of (Q)SAR systems for mammalian cell genotoxicity assays is inadequate coverage of

Comment [.8]: IGG feels this infers to great weight on the SAR. WP CONSIDERED

non-covalent DNA interactions and non-DNA targets associated with cell division (Grant et al., 2000, Snyder and Smith, 2005). It has also been proposed that (Q)SAR assessments can aid in the interpretation of the relevance of *in vitro* genotoxicity assays through prediction of biotransformation (Combes et al., 2007). Other systems combining metabolic simulation with structure toxicity rules have been developed (e.g TIMES; tissue metabolic simulator) but are at a relatively early stage of validation (Mekenyan et al., 2004, Serafimova et al., 2007). Lhasa Ltd has~~ve~~ developed a computer programme (METEOR), which has the facility to integrate prediction of metabolism with (Q)SAR approaches for genotoxicity. (https://www.lhasalimited.org/meteor/)

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

~~28. A compilation of structural alerts for prediction of the rodent *in vivo* micronucleus assay has recently been published. The authors advocate that the derived rules can be used for preliminary identification of *in vivo* mutagens (Benigni et al., 2010).~~23. Overall, (Q)SAR approaches for the prediction of genotoxic activity can be a valuable tool to aid in the high throughput screening of compounds, the provision of assessments for chemicals for which no genotoxicity~~ology~~ test data are available and also prioritisation for

genotoxicity testing. (Q)SAR can also aid in the interpretation of genetic toxicology tests, although such predictions cannot replace the need to undertake the *in vitro* and *in vivo* genotoxicity tests ~~currently~~ required to derive conclusions on mutagenic hazard. With regard to chemicals for which there are limited, possibly inadequate, or no genotoxicity test data available, a (Q)SAR prediction of mutagenicity (within the domain of applicability of the system(s) used) should be taken as preliminary evidence for potential or lack of potential mutagenicity. However, expert judgement is needed when reaching conclusions on mutagenic hazard on the basis of (Q)SAR information alone. In reaching conclusions, data from well conducted *in vitro* or *in vivo* genotoxicity tests should be attributed a much higher weight of evidence than (Q)SAR predictions, although all information should be assessed on a case-by-case basis.

Screening Tests

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

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25. A number of genotoxicity screening tests using *in vitro* systems have been proposed, including~~,~~ alkaline elution using rat hepatocytes (Gealy et al., 2007), the detection of DNA damage (via p53 or GADD45a activation, GreenScreen), in cell lines (Knight et al., 2009) and differential survival ~~growth~~ in DNA repair proficient and deficient cell lines (Helleday et al., 2001). A screening test for genotoxicity using HepG2 (metabolically competent with wild type p53 genotype) based on four different luciferase-reporter assays has been published. The authors claim, based on a small dataset, a high sensitivity for prediction of genotoxicity when used in combination with the commercially available systems (Vitotox™ and RadarScreen) (Westerink et al., 2010). None of these genotoxicity screening tests have reached the stage of development where they could routinely be used to replace data generated from *in vitro* genotoxicity testing. The predominant use of high throughput screening tests is as an aid in prioritisation of compounds for development undertaken by industry. The COM~~ommittee has~~ reviewed the ~~agreed that the~~ GADD45a-GFP assay and it was agreed that currently, it is most suited as part of a battery of high throughput screening (COM minutes March 2010, http://www.iacom.org.uk/meetings/index.htm).

26~~7~~. High throughput genotoxicity screening tests can be used in a tiered approach with *in vitro* genotoxicity tests ~~during chemical development~~to aid in the selection of chemicals for development. It has been suggested that greater validation and acceptance by regulatory authorities of these tests could lead to the replacement of existing genotoxicity testing strategies with a combination of high throughput screening tests (Custer and Sweder, 2008).

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

Overview of strategy

27. The COM concluded in 1989 and 2000 that it was appropriate to concentrate on a relatively small number of assays, using validated, sensitive methods particularly chosen to ***avoid misleading negative results***. Two important parts of the revised Stage 1 strategy include using appropriate tests to gain an insight into the nature of the genotoxic effects of a test substance and also to

Comment [.9]: SFTG/EEMS : comment that reference to sensitivity/specificity should be more clearly stated throughout.

avoid misleading positive results. Misleading positive results have been reported for certain mammalian cell assays (Kirkland et al., 2007a, Fowler et al., 2009a, Pfuhler, 2009)~~(Kirkland et al., 2007a, Pfuhler, 2009)~~ particularly when multiple test systems were used~~(Fowler et al., 2009a)~~.

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28. As outlined above in paragraph 13, Stage 1 involves tests for genotoxic activity using *in vitro* methods and comprises a two test core system (namely an Ames test and *in vitro* micronucleus test (MNvit)) with the objective of assessing mutagenic potential by investigating three different end points (gene mutation, structural chromosomal damage and changes in chromosome number). The rationale for this is given in Annex 3. A clear positive result in either of these two core tests is sufficient to define the chemical as an *in vitro* mutagen, although further *in vitro* and/or *in vivo* testing may be undertaken to understand the relevance of the positive results. The Committee considers that this revised strategy allows for efficient identification of all mutagenic end points but, by reducing the number of mammalian cell tests from that recommended by COM in 2000, and following improved methodologies, the risk of misleading po~~ with an optimal low level of misleading po~~sitive results is decreased.~~.~~ ~~The rationale for this is given in Annex 3.~~

29. Additional investigations of chemicals which give positive or repeated equivocal results in Stage 1 tests can include an assessment of mode(s) of *in vitro* genotoxic action (MoGA). There are a number of reasons (discussed in paragraphs 37-40 ~~X~~ ~~below~~) why positive results in *in vitro* genotoxicity tests might occur by mode(s) of action not relevant to human health hazard assessment. Such MoGA evaluation *in vitro* is particularly relevant for those chemicals (e.g. cosmetics) where there is a regulatory constraint which precludes the use of *in vivo* genotoxicity assays in the testing strategy. The COM does not recommend the use of in *vitro* genotoxicity assays that have not been considered in detail ~~cited~~ in this guidance such as assays for sister chromatid exchange, the *in vitro* UDS assays or tests using fungi. A table of mutagenic endpoints detected by each genotoxicity assay cited in Stage 1 of this strategy is given in Annex 2.

30. For chemicals which give equivocal results or repeated small positive effects, it is important to consider evidence of reproducibility in the same assay or in

different assays detecting similar effects, and the magnitude of the induced genotoxic effect in relation to historical negative control data, and then consider whether further *in vitro* genotoxicity testing is needed (Kirkland et al., 2007b, Hayashi et al., 2010) . Further consideration of ~~of MoGA and~~ SAR data for these chemicals may ~~can~~ also give valuable information (Dearfield et al., 2010).

31. I~~n general, i~~f clear negative results are obtained in both core ~~all~~ *in vitro* tests undertaken, it can generally be concluded that the chemical has no mutagenic activity. However, there are some occasions when additional *in vitro* and/or *in vivo* genotoxicity testing may be undertaken for chemicals giving a negative response in the two *in vitro* core genotoxicity tests for example, where tumours are ~~subsequently~~ found in rodents and specific rodent or human metabolites or where *in vitro* metabolic activation systems may not be optimal, need to be subject to further genotoxicity assessment~~, or the test substance has a structural alert but *in vitro* genotoxicity tests were negative~~. A further testing strategy would have to be designed on a case-by-case basis (Muller et al., 2003a, Kirkland et al., 2007b). An IWGT working group has published guidance on this topic (Kasper et al., 2007). An important part of any additional *in vitro* strategy should be consideration of the appropriate exogenous metabolic activation system (including alternative sources of S-9 or other metabolic systems including genetically engineered cell lines)(Ku et al., 2007b). Further information on *in vivo* genotoxicity testing of such test substances is provided in Stage 2 of this strategy.

32. Information from other combinations of genotoxicity tests which may include one or more non-core tests outlined below in paragraphs 55-60~~7-59~~ (and in Figure 2) may also give adequate data on all three end-points on a case-by-case basis. *In vitro* genotoxicity tests using human reconstructed skin may provide useful information on *in vitro* mutagenic hazard in circumstances where *in vivo* testing is not permitted, or when extensive dermal exposure is anticipated (e.g. ~~for example with~~ cosmetic ingredients).

33. The full Stage 1 strategy should be performed and the results of studies evaluated before a decision is made on whether to proceed to Stage 2 testing or whether a conclusion on mutagenic hazard can be derived for test

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Comment [.10]: Sentence removed following discussion of GUM comment re negative results overulling SAR

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Comment [.11]: Phys Committee: enlarge to include chemicals with extensive dermal contact, provide evidence of dermal metabolism?
WG considered that this covered it.

substances where no *in vivo* genotoxicity testing is permitted. An outline of Stage 0 and Stage 1 (*in vitro* genotoxicity testing) is given in Figure 2 and a description of the assays recommended is provided in the following paragraphs.

Discussion of Stage 1 Tests- General Aspects

34~~5~~. The conduct of genotoxicity assays has improved over time and the overall sensitivity of *in vitro* testing strategies regarding prediction of rodent carcinogens is very high (Kirkland et al., 2005a, Kirkland et al., 2007a)~~(Kirkland et al., 2007a)~~. Proposals have been published for genotoxicity testing advocating a single *in vitro* genotoxicity test (Ku et al., 2007a) or a complex approach involving up to six *in vitro* genotoxicity tests (SCCNFP/0720/03) and critically evaluated by Kirkland et al (Kirkland et al., 2005b)~~Kirkland et al., 2005b)~~. Neither of these approaches is considered preferable to the proposed Stage 1 core testing. Although the sensitivity for rodent carcinogenicity of the Stage 1 tests was ~~is~~ very high, the specificity (producing negative results with non-carcinogens) was~~is~~ poor ~~-~~(Kirkland et al., 2005a, Kirkland et al., 2007b, Kirkland et al., 2007d)~~(Kirkland et al., 2007d)~~. Possible reasons for the poor specificity have been discussed by various working groups e.g., see (Kirkland et al., 2007b)~~Kirkland et al 2007d~~. A comprehensive review of the performance of Stage 1 genotoxicity assays for prediction of rodent carcinogenicity reported positive results in one or more *in vitro* tests for a substantial number of rodent non-carcinogens (as assessed by the Carcinogenic Potency Database (CPDB), National Toxicology Program (NTP), and the International Agency for Research on Cancer (IARC)). Thus the specificity (i.e. correct identification of rodent non-carcinogens) was considered to be reasonable for the Ames test (74%) but poor for the mammalian cell assays (below 45%) particularly when multiple assays were performed (Kirkland et al., 2005a)~~(Kirkland et al., 2005a)~~(Elespuru et al., 2009). Many reasons for low specificity have been proposed, particularly for mammalian cells; for example, high-concentrations, cytotoxicity, prolonged exposure, overloading defence mechanisms, lack of detoxification capacity. The influence of such confounding effects has been widely recognised (Müller and Kasper, 2000, Kirsch-Volders et al., 2003b, Pratt and Barron, 2003)

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Comment [.12]: Several comments: include more explanation of spec/sensitivity of mammalian cell assays, Accuracy of predictions for carcinogenicity?

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35. A more recent analysis ~~Data on~~on the sensitivity of ~~, including the~~a combination of Ames test and *in vitro* micronucleus test to ~~for the~~ detect~~ion of~~ rodent carcinogens and *in vivo* genotoxicants is summarised in~~ns, are given in~~ Annex 1, Table 4, discussed in Annex 3 and published in Kirkland et al (Kirkland et al., 2011). It is difficult to draw precise conclusions from these data since the databases of chemicals used vary. However these data do show that mammalian cell genotoxicity tests can have low specificity and that combinations of *in vitro* genotoxicity tests result in high sensitivity for rodent carcinogens and *in vivo* genotoxicants. H~~A h~~igh sensitivity has always been a ~~has been a~~ priority of ~~previous~~ genotoxicity testing strategies recommended by the COM (DOH, 2000)~~(DOH., 2000)~~. An evaluation of the use of *in vitro* genotoxicity tests to predict rodent carcinogens and *in vivo* genotoxicants~~ns~~ prepared for the COM meeting in June 2010 (http://www.iacom.org.uk/papers/index.htm MUT/2010/08) concluded that there is no convincing evidence that any rodent carcinogen or *in vivo* genotoxicant ~~n~~ would fail to be detected ~~be "missed"~~ by using an *in vitro* genotoxicity test battery consisting of Ames test and *in vitro* micronucleus test. ~~Further rationale and justification for using this combination of *in vitro* tests are provided in Annex 3.~~

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36. It is most likely that the few occasions where *in vitro* test strategies fail to detect mutagenic activity (i.e. misleading negative results) will be due to the absence of appropriate metabolic activity *in vitro* (Brambilla and Martelli, 2004). Approaches to resolving potential inadequacies in metabolic activation include structure based metabolism predictions, use of genetically modified target organisms (e.g. CYP2E1 in *Salmonella* YG7108pin3ERb$_5$) (Emmert et al., 2006), the use of exogenous metabolic activation systems derived from human sources, or recombinant human cytochrome P450 systems as an external activation system (Ku et al., 2007b).

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37. There are a number of ~~non DNA interaction~~ MoGAs by which a chemical may demonstrate an *in vitro* genotoxic effect that is either not relevant for humans or has a threshold. The COM has reviewed the evidence for a number of threshold MoGAs and a general guidance statement is available.

(http://www.iacom.org.uk/guidstate/index.htm statement G05). Threshold MoGAs can generally be considered as non-DNA interactions or an overload of normal cellular physiology'. In such ~~both~~ cases a No Observed Effect Concentration (NOEC) can be determined and may be useful in evaluating risk. ~~The COM has reviewed the evidence for a number of threshold MoGAs and a general guidance statement is available (http://www.iacom.org.uk/guidstate/index.htm statement G05). Threshold MoGAs can be generally be considered as an overload of normal cellular physiology'.~~ Investigations of MoGA need to be designed on a case-by-case basis and can be complex to interpret (Kirkland et al., 2007a).

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38. There has been considerable debate regarding the highest concentration that should be used routinely in mammalian cell assays. The International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is considering whether the maximum concentration tested for pharmaceuticals should be 1mM in mammalian cell genotoxicity assays which would have the effect of reducing the number of misleading positive results due to excessive concentrations where the cellular defence mechanisms might be overwhelmed. However a reduction to 1mM would not be consistent with the OECD recommendation for a top concentration of 10mM in mammalian cell genotoxicity assays (OECD, 1997)~~(OECD., 1997)~~. A recent analysis of published data for the top concentration in mammalian cell genotoxicity tests identified a small number of carcinogens that (according to the publications) would not be detected in any part of a three test *in vitro* genotoxicity test battery (consisting of the Ames, mouse lymphoma and *in vitro* chromosomal aberration tests) if the testing concentration limit for mammalian cell assays were reduced from 10mM to 1mM (Parry et al., 2010) A further investigation of these carcinogens found that some positive results at concentrations above 1mM were not reproducible (i.e. they were not genotoxic in mammalian cells under current OECD guideline protocols) and others were positive at concentrations below 1mM, particularly when continuous treatments in the absence of S-9 (not included in the original publications) were conducted. A ~~Thus a~~ new upper limit for mammalian cells tests of 1mM or 500 µg/ml (whichever is

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higher) has been proposed as sufficient to detect all genotoxic carcinogens that are negative in the Ames test (Kirkland and Fowler, 2010). Several international organisations are examining the principles underpinning this upper limit selection (e.g ICH, OECD, IGWT) although currently there no international consensus has been reached .~~Thus the available evidence supports an upper concentration limit of 1mM for mammalian cells but there is a need to reach international consensus on this proposal before making a recommendation on its application to genotoxicity tests using mammalian cells~~.

39. There has also been considerable investigation of the role of excessive cytotoxicity in mammalian cells and choice of cell type as possible causes of misleading positive results (Blakey et al., 2008, Fellows et al., 2008b, Pfuhler, 2009)~~(Blakey et al., 2008, Fellows et al., 2008b, Pfuhler, 2009)~~. The method used to assess cytotoxicity may affect the selection of highest concentration tested and potentially the results obtained using mammalian cell genotoxicity assays (Kirkland et al., 2007d) and recommendations have been made to use ~~-~~ cytotoxicity measures based on cell proliferation (Galloway and Levy, 2011). However, it is important to note that although excessive cytotoxicity may lead to misleading positive results, it may also result in misleading negative results when pronounced cell cycle delay occurs. A similar conclusion was reached at an international symposium on regulatory aspects of genotoxicity testing (Blakey et al., 2008)~~B(Blakey et al., 2008)~~).

40. Most ~~any~~ cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems. These cell lines also have impaired p53 function and altered DNA repair capacity (Kirkland et al., 2007d). There is some evidence that human lymphocytes are less susceptible to misleading positives than the rodent cell lines currently used (e.g. CHO, V79 CHL and V79). Other cell systems such as the human cell lines HepG2, TK6 and M~~CL~~-5 cells and the reconstructed human skin models and HepaRG show promise for future use (Kirkland et al., 2007d, Fowler et al., 2009b, Le Hegarat et al., 2010)~~(Kirkland et al., 2007d, Fowler et al., 2009b)~~.

Comment [.13]: GUM ask : how would the 2 battery perform if top conc was reduced to 1mM – CAN'T BE ANSWERED

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Comment [.14]: Mention Precipitation

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41. The COM agrees that it is not necessary to undertake independent confirmatory *in vitro* tests when clear negative or positive results have been obtained provided the following criteria are satisfied:

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

42~~3~~. It is recognised that it can be difficult to provide convincing evidence for ~~an~~ absence of ~~a~~ genotoxic effects ~~(i.e. a consistently clear negative result)~~. The investigator should consider the power of the study design and the past performance of the test system when formulating a protocol in order to optimise the chances of obtaining an unequivocal result from a single experiment and to ensure that any potential genotoxic effect is not missed.

43. There is a need to undertake further *in vitro* genotoxicity testing when an equivocal result is obtained (i.e. neither clearly negative nor clearly positive by appropriate biological or statistical criteria). Such additional genotoxicity tests need to be planned on a case-by-case basis and need not necessarily be undertaken in an identical fashion to the initial experiment(s). Indeed it may be preferable to alter certain aspects of the study (e.g. concentration levels investigated, treatment and sampling times, concentration of metabolic activation mix) so as to obtain supplementary data. It may also be appropriate to use a different genotoxicity test system, e.g. a chromosomal aberration test, if there is equivocal evidence of clastogenicity from an *in vitro* micronucleus test, or an *in vitro* cell mutation assay (e.g. TK or ~~mouse lymphoma mutation assay~~HPRT mutation assays) if there is equivocal evidence of gene mutations from an Ames test.

44~~5~~. The use of historical negative control data to aid in the interpretation of genotoxicity test results has been considered particularly in relation to

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equivocal and small magnitude genotoxic effects (Kirkland et al., 2007b). Advice has been recently published on approaches to collecting historical control data. Ideally data should be reported in terms of means and confidence intervals for the distribution of baseline genotoxic effects rather than observed ranges where outliers can have a disproportionate effect. The dataset should be updated regularly and should be as large as possible. Negative ~~H~~historical ~~negative~~ control data should have been generated using a fixed testing protocol unless it can be demonstrated that changes in protocol do not impact on the range of values reported in studies (Hayashi et al., 2010). The Committee considers that in some instances negative historical control data can be a valuable aid in the interpretation of genotoxicity tests.

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

Discussion of Stage 1 strategy: Specific Core Tests

In Vitro Bacterial Tests for Gene Mutations

46. The most widely used *in vitro* mutagenicity test is the bacterial reverse mutation assay for gene mutations developed by Ames and his colleagues using *Salmonella typhimurium* (Gatehouse et al., 1994). The very extensive database available for this assay justifies its inclusion in any initial genotoxicity testing for mutagenic hazard. Several strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the best validated strains being TA-1535, TA1537 (or TA97 or TA97a), TA98 and TA100. In addition , ~~These strains of *Salmonella typhimurium* may not detect some oxidising mutagens and cross linking agents and thus~~ *Escherichia coli* WP2*uvrA* or WP2 *uvrA*(pKM101)~~, WP2*uvrA*~~ or *Salmonella* TA102 are included in the standard tester strain. In order to detect oxidising and cross-linking agents set TA102 should be included or a repair proficient *Escherichia coli* strain (WP2 or WP2 (pKM101)). ~~should also be used.~~ Testing should be carried out both in the presence and absence of an appropriate exogenous

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Comment [.18]: IGG request clarification . Should we be more explicit in what is required? WP CONSIDERED BUT REJECTED THIS

Comment [.19]: IGG note that this paper is likely to raise discussion in this area.

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metabolic activation system such as S-9. Both plate-incorporation and pre-incubation methods are widely used and should be considered.

4~~7~~7. There have been developments to automate and minimise the amount of test substance required for the Ames test (e.g. Spiral *Salmonella* mutagenicity assay (Claxton et al., 2001) and Ames II™ test (Fluckigetr-Isler et al., 2004)). The Committee considers that these methods have not been developed to a point where they can be routinely ~~be~~ used for regulatory submissions.

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

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

49. The *in vitro* micronucleus test can be carried out in the absence or presence of cytochalasin B, which is used to block cell division and generate binucleate

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Comment [.22]: Xenometrix say Ames II has been used in regulatory submissions, this reflects the WP POSITION.

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cells (CBMN method). The advantage of using cytochalasin B is that it allows clear identification that treated and control cells have divided *in vitro* and provides a simple assessment of cell proliferation. The use of cytochalasin B has no impact on the sensitivity of the test results (Garriott et al., 2002, Lorge et al., 2006, Oliver et al., 2006, Wakata et al., 2006). ~~Micronuclei are scored in mononucleated cells when experiments are performed in the absence of cytochalasin B.~~ The target population in the presence of cytochalasin B are~~is~~ the binucleate cells (because it is clear they have divided);~~,~~ however scoring of both mononucleated and binucleated cells can be useful for the detection of aneugens (Lorge et al., 2006, Wakata et al., 2006). In the absence of cytochalasin B, it is essential to have evidence that cells have divided.

50~~1~~. There have been major international collaborative investigations to develop the protocol (Garriott et al., 2002, Phelps et al., 2002, Kirsch-Volders et al., 2003a, Lorge et al., 2006)~~(Garriott et al., 2002, Phelps et al., 2002, Kirsch-Volders et al., 2003, Lorge et al., 2006)~~, provide information on the performance of this assay using different cell lines (Oliver et al., 2006, Wakata et al., 2006, Fowler, 2009, Pfuhler, 2009)~~(Oliver et al., 2006, Wakata et al., 2006, Fowler, 2009, Pfuhler, 2009)~~, to investigate the most appropriate methods for measuring cytotoxicity (Fellows et al., 2008a, Lorge et al., 2008, Kirkland, 2010) and initial studies to evaluate a flow cytometric approach to the micronucleus assay (Bryce et al., 2007, Bryce et al., 2008a, Laingam et al., 2008). The *in vitro* micronucleus assay can be performed using most mammalian cell lines used in genotoxicity testing (Lorge et al., 2006). However there is emerging evidence that rodent cell lines with compromised p53 activity such as V79, CHO and CHL cells can give more misleading positive results than cell lines proficient for p53 activity such as TK6 a~~HepG2 a~~nd human lymphocytes (Fowler, 2009, Fowler et al., 2009a)~~(Fowler et al., 2009a)~~. Overall the COM's preference is for human lymphocytes which have a number of advantages over cell lines (e.g. normal diploid primary human cells with some protection against oxidative damage when whole blood cultures are used). If cell lines are used, it is important that the impact of potential genetic drift of the cells cultured is understood (Tweats et al., 2007a)

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51~~2~~. There have been considerable developments on deriving suitable protocols for the *in vitro* micronucleus assay using both cell lines and lymphocytes (Garriott et al., 2002, Phelps et al., 2002, Kirsch-Volders et al., 2003a, Aardema et al., 2006, Clare et al., 2006)~~(Garriott et al., 2002, Phelps et al., 2002, Kirsch-Volders et al., 2003, Aardema et al., 2006, Clare et al., 2006)~~. One particular area of protocol development which has been subject to considerable investigation is the most appropriate method(s) for estimating cytotoxicity in *in vitro* micronucleus tests (Fellows et al., 2008a, Lorge et al., 2008, Kirkland, 2010). It has been suggested that using relative cell counts (RCC) may underestimate cytotoxicity and lead to potentially misleading positive results (Fowler et al., 2009b). In the absence of cytokinesis block, the relative increase in cell count (RICC) or relative population doubling (RPD) are comparable with replication index (RI) used with the cytokinesis block assay and are the most appropriate methods of cytotoxicity estimation. It has been suggested that testing beyond 50% survival is not necessary to identify potential mutagens (Fellows et al., 2008b, Lorge et al., 2008)~~(Fellows et al., 2008b, Lorge et al., 2008)~~. However consensus recommendations embedded in the OECD guideline 487 indicate that the target range for cytotoxicity in the *in vitro* micronucleus test is 55±5%. Careful selection of toxicity measure has been shown to reduce the potential for misleading positive results (Fowler et al., 2009b).

52. The *in vitro* micronucleus assay can be combined with centromere or kinetochore stains, with pancentromeric or chromosome specific centromeric probes using fluorescence *in situ* hybridisation (FISH) as a sensitive way to discriminate between chromosome breaks, chromosome loss and chromosome non-disjunction and polyploidy (Kirsch-Volders et al., 2002) and therefore is useful in assessing mode of action (Parry, 2006). Binucleate cells obtained with the cytokinesis block methodology (CBMN) will usually be needed for determination of non-disjunction of chromosomes between daughter nuclei. Fenech has proposed that the CBMN assay can be further modified to provide comprehensive information on nucleoplasmic bridges (NPBs) which may provide information on chromosome rearrangements or telomere end fusions, and nuclear buds (NBUDs) which may provide information on gene

amplification (Fenech, 2006, 2007). Fenech proposed that the comprehensive CBMN assay should be considered as a 'cytome' method for measuring chromosomal instability and altered cellular viability (Fenech, 2006). The 'cytome' method is complex and requires considerable technical skill and is currently not suitable for routine testing of chemicals for genotoxicity but may provide useful information on MoGA. ~~.~~

53. The flow-cytometry-based micronucleus assay (FCMMN) has the potential for increased reproducibility and decreased turnaround time for the micronucleus test (Laingam et al., 2008)~~(Laingam et al., 2008)~~. However the potential still exists for misleading positive results from cell processing or from chemical induced apoptosis and necrosis (Laingam et al., 2008). Approaches to overcoming potential misleading positive results have included: the use of differential staining of micronuclei (MN) and necrotic and apoptotic cells, (Bryce et al., 2007, Bryce et al., 2008a), the use of electronic gating procedures and the use of concurrent assessment of cytotoxicity (Laingam et al., 2008). The FCMMN assay has also been adapted to cell lines which attach to solid surfaces (Bryce et al., 2010). The COM acknowledges the ongoing ~~considers that further~~ validation of the *in vitro* FCMMN assay which is important ~~required~~ before it can be used for regulatory submissions. A separate approach to automation of the CBMN assay involves automated image analysis using Giemsa stained slides (Decordier et al., 2009) which may be useful with appropriate validation.

Discussion Stage 1: Non-Core Tests

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

54~~5~~. The *in vitro* chromosome aberration assay in mammalian cells has been widely used in genotoxicity testing for many decades and provides information on genetic damage that may be associated with adverse health outcomes. Only limited information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (Aardema et al.,

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1998). The COM notes that polyploidy may not be a reliable indicator for aneugenicity and may result from a number of different genetic changes (Mitchell et al., 1995, Galloway, 2000)~~(Mitchell et al., 1995 , Galloway, 2000).~~ ~~However the *in vitro* chromosomal aberration assay may provide information on exchanges which are associated with adverse health outcomes.~~ It is possible to ~~It is important to~~ adapt the chromosome aberration assay to include the use of chromosome specific centromeric probes with fluorescence *in situ* hybridisation (FISH) to assess the potential for aneuploidy~~. A wide range of FISH technologies exist for the analysis of clastogenic and aneugenic chromosome changes~~ (Maierhofer et al., 2002). ~~One published evaluation of *in vitro* genotoxicity testing strategies reported that there was no scientific basis for including both a chromosomal aberration and micronucleus assay in addition to Ames and mouse lymphoma assays (Kirkland et al., 2005b).~~ An IWGT working group ~~(Galloway et al., 2010)~~ (Galloway and Levy, 2011) has agreed that the preferred measure of cytotoxicity in the chromosomal aberration test should be one based on cell proliferation (e.g. relative population doubling or relative increase in cell counts) compared to ~~in~~ negative control cultures rather than simple cell counts. The available data indicate that the *in vitro* metaphase analysis and the *in vitro* micronucleus a~~a~~ssay have similar overall performance for determination of ~~as part of~~ clastogenicity. ~~a strategy for genotoxicity testing.~~ On balance it is considered preferable to use the *in vitro* micronucleus test for the initial assessment of clastogenic and aneugenic potential.

In Vitro Mouse Lymphoma Assay for Gene Mutation and Clastogenicity

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

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56~~7~~. Since 2000, there has been considerable development of suitable protocols, negative solvent control data, criteria to define an acceptable positive control response and the use of the Global Evaluation Factor (GEF) and statistical analysis of test results (Clements, 2000, Moore et al., 2003, Moore et al., 2006, Kirkland et al., 2007c, Moore et al., 2007)~~(Clements, 2000, Moore et al., 2003, Kirkland et al., 2007c, Moore et al., 2007)~~. Many of the published studies were undertaken by the US National Toxicology Program (NTP) and a recent re-evaluation of these results shows many of the studies to be uninterpretable or the outcomes to be equivocal (Schisler et al., 2010). Some authors have reported that the mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity_, information on recombination, deletion and aneuploidy (Sofuni et al., 1996, Ogawa et al., 2009, Wang et al., 2009)~~(Ogawa et al., 2009, Wang et al., 2009)~~. It is possible that aneuploidy in these cells could be a secondary effect of chromosomal rearrangement. However the COM considers that this assay is not appropriate for the routine assessment of aneuploidy.

In vitro HPRT assays for Gene Mutation

57~~8~~. An *in vitro* cell mutation assay which uses forward mutation in the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene to assess mutations has been developed in several cell lines, principally Chinese hamster ovary cells (CHO) cells (Li et al., 1988). It is described in OECD 476 guideline. The Committee have previously considered this http://www.iacom.org.uk/meetings/02.10.2003.htm. However certain mammalian cell gene mutation protocols that have been widely used, particularly some involving CHO cells and the use of these are considered to be insufficiently sensitive for the identification of mutagens, predominantly on statistical grounds (UKEMS., 1989).

In Vitro Assays using Human Reconstructed Skin

58~~9~~. A number of research groups have developed genotoxicity assays based on micronuclei measurement using commercial sources of human reconstructed skin (such as Episkin® and EpiDerm™) (Curren et al., 2006, Flamand et al.,

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2006, Hu et al., 2009, Mun et al., 2009) or a co-culture technique involving reconstructed skin and mouse lymphoma L5178Y cells (Flamand et al., 2006). Proposals for the measurement of DNA damage using the C~~c~~omet assay in reconstructed skin have also been made (Pfuhler, 2009)~~(Pfuhler, 2009)~~. The primary purpose in developing genotoxicity tests using reconstructed skin has been to supplement genotoxicity data-packages for cosmetic chemicals where no *in vivo* genotoxicity tests are permitted. A tiered approach to testing cosmetic ingredients for genotoxicity has recently been published (Pfuhler et al., 2010).

In Vitro Alkaline Comet Assay for DNA Damage

59~~60~~. The *in vitro* alkaline C~~c~~omet assay for DNA damage has been proposed as an alternative to clastogenicity assessment in mammalian cells since cell proliferation is not needed, therefore any cell type can be used, and the assay is reported to result~~s~~ in fewer misleading positive results due to cytotoxicity or precipitation than chromosomal aberration tests (Hartmann et al., 2001, Witt et al., 2007). The alkaline C~~c~~omet assay detects a wide range of genetic damage including single and double strand breaks, repair induced breaks, alkali labile lesions and abasic sites. There is evidence that the *in vitro* C~~c~~omet can be used to detect DNA cross-linking agents (Spanswick et al., 2010). The ~~comet~~Comet-FISH assay has been recently developed to provide information on site specific DNA strand breaks (Glei et al., 2009). There is evidence that the *in vivo* ~~comet~~Comet assay can detect substances that induce gene mutations *in vitro* (Kirkland and Speit, 2008, Kawaguchi et al., 2010)~~(Kirkland and Speit, 2008)~~. ~~-~~Extrapolation from this would suggest that the *in vitro* ~~comet~~Comet assay can also detect substances that induce gene mutations . However it is not recommended as a replacement for gene mutation tests *in vitro*. Thus the ~~comet~~Comet assay measures DNA damage irrespective of genotoxic end-point ~~MoGA~~, with the exception of aneuploidy. A positive ~~comet~~Comet assay result may be due to repairable DNA damage or lesions which lead to cell death and not necessarily mutations or micronuclei. Negative results from an Ames test and MNvit would reduce the level of concern associated with positive results from an *in vitro* ~~comet~~Comet assay. Thus, the *in vitro* ~~comet~~Comet assay can serve as a very useful adjunct to the

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Comment [.32]: SFTG EEMS: comment that in vi comet has less false positives that CA

recommended core-tests, especially in instances where *in vivo* testing is not permitted such as in cosmetic testing. However, since the ~~comet~~Comet assay does not detect aneuploidy, and may report repairable DNA damage, it is not recommended as a core *in vitro* test.

Summary Stage 1 (*In Vitro* Genotoxicity Testing)

60~~1~~. The COM recommendations for Stage 1 testing incorporate a number of changes to the 2000 guidelines, the main changes being the replacement of the *in vitro* metaphase analysis in mammalian cells with the *in vitro* micronucleus assay and a reduction from three tests to two *in vitro* tests for Stage1. Tests should be undertaken according to the best international guidance available to avoid misleading positive or negative results. Data should be interpreted using appropriate statistical analysis and use of historical negative control data. The COM confirms the need to provide information on gene mutation, clastogenicity and aneugenicity and~~in order~~ to understand genotoxic mode(s) of action (MoGA) in order ~~and~~ to derive conclusions regarding the biological importance of results. Data on MoGA are important in elucidating whether genotoxicity tests give misleading negative or positive results, and also to aid decisions with regard to devising a strategy for Stage 2 *in vivo* genotoxicity testing. There is a particular need to understand MoGA for chemicals which cannot be subjected to *in vivo* genotoxicity tests (e.g. cosmetics). In this particular instance some useful additional information on genotoxicity may be provided by undertaking *in vitro* tests using reconstructed human skin. The recommended two core genotoxicity tests in Stage 1 are the *in vitro* bacterial gene mutation test and in *vitro* micronucleus test (MNvit). These recommended assays, when combined, provide sufficient information for the genotoxicity assessment of most chemicals and provide high sensitivity for the detection of rodent carcinogens and *in vivo* genotoxicants, and reduce the risk of misleading positive results. Information from non-core tests described in this document may provide useful additional information on *in vitro* mutagenic hazards on a case-by-case basis. In most instances misleading negative *in vitro* results are due to inadequate exogenous metabolic activation (Ku et al., 2007b). However, ~~in~~ some ~~instances~~ regulatory authorities may require an *in vivo* genotoxicity test where high, or moderate and prolonged,

levels of exposure are expected (e.g. most human medicines) in order to provide additional reassurance even when Stage 1 tests have given negative results. If a chemical is considered on the basis of Stage 1 test results to have *in vitro* mutagenic potential but has not been tested *in vivo,* the COM considers it prudent to assume that the chemical may have *in vivo* mutagenic potential.

> **Comment [.33]:** HSE comments that this does not capture the situation where other data are used in the genotoxicity assessment. WP NOT RELEVENT HERE

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

Overview of Strategy

61~~2~~. Stage 2 of the testing strategy involves an assessment of genotoxic activity *in vivo* in somatic tissues and in germ cells (when there is a need for the assessment of heritable effects and/or information on hazard classification of mutagens) (see Figure 3). The *in vivo* genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to address the following aspects of *in vivo* mutagenicity;

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

> **Comment [.34]:** In response to a comment from GUM

It is thus possible for there to be one or more separate Stage 2 strategies designed to assess points 1)-5~~4~~) for a particular test substance. This rationale leads to different approaches from those advocated by the COM in 2000 where the weight of available evidence suggested that the *in vivo* bone marrow (or peripheral blood) micronucleus assay or bone marrow clastogenicity assay in rodents was the preferred first test in almost all cases. The exception was for direct acting DNA reactive mutagens where a site of contact test was preferred. There was a preference in the 2000 COM guidance for the rat liver UDS assay as a second tissue *in vivo* test, which was selected primarily to provide reassurance of absence of *in vivo* genotoxicity when positive results had been obtained *in vitro* but negative results were obtained in an *in vivo*

bone marrow micronucleus or chromosomal aberration assay. The selection of rat liver UDS was based largely on experience in use and the availability of an OECD guideline (DOH, 2000)~~(DOH., 2000)~~. The ~~A~~ revised *in vivo* Stage 2 strategy based on the selection of tests to provide information on one or more specific aspects such as species and/or tissue genotoxicity combined with investigation of particular genotoxic end points and modes of genotoxic action does ~~would~~ not necessarily lead to the selection of rodent bone marrow micronucleus test as the first assay or the rat liver UDS assay as a second tissue assay.

62~~3~~. Other factors that should be considered when determining an *in vivo* genotoxicity testing strategy include whether the testing strategy can be integrated into other regulatory toxicity tests (such as subacute or subchronic toxicity studies). Consideration needs to be given to the nature of the chemical (including physico-chemical properties), the results obtained from *in vitro* genotoxicity tests and the available information on the toxicokinetic and metabolic profile of the chemical (for example when selecting most appropriate species, tissue and end point). The routes of exposure in animal studies should be appropriate to ensure that the substance reaches the target tissue. Routes unlikely to give rise to significant absorption in the test animal should therefore be avoided. Evidence for exposure of the target tissue to the test chemical or its metabolites should be provided before a negative conclusion can be reached. Unless systemic exposure can be confirmed from other toxicological studies, or evident toxicity in the target organ is seen, confirmatory toxicokinetic studies to measure blood or tissue exposure as appropriate should be undertaken to accompany all *in vivo* genotoxicity studies to assess the adequacy of any negative results obtained.

63~~4~~. The design of *in vivo* genotoxicity tests should incorporate appropriate approaches to reduce the number of animals used in such tests such as the integration of genotoxicity endpoints into repeat-dose studies. Options for reduction in animal usage include:

- u~~U~~se of one sex only (if supported by metabolism data or other data indicating equivalence),

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Comment [.35]: More guidance on exposure ? Should we refer to data from 28 d studies? IGG WP -THINK WE'VE COVERED THIS

- reduced numbers of sampling times for micronucleus and chromosomal aberration assays when repeat dosing is performed,
- integration of micronucleus and comet end points into repeat-dose toxicity (including transgenic mutation) studies,
- combining micronucleus and ~~comet~~Comet assays into a single acute test employing a few administrations of test chemical (Pfuhler et al., 2009, Bowen et al., 2010, Vasquez, 2010, Bowen et al., 2011),

64. It should also be possible to omit the concurrent positive control administrations in micronucleus and chromosomal aberration tests (but not for the Comet assay) where the test facility has appropriate historical positive control data (Pfuhler et al., 2009) as long as positive control slides "banked" from previous treatments and coded in with the experimental slides, are included to demonstrate proficiency.

65. The toxic properties of test substances (such as acute toxicity, subchronic toxicity (including target organ effects), irritancy/corrosivity in contact with skin or mucous membranes), toxicokinetic and metabolism data will influence the choice of route of administration and the highest dose level achievable in *in vivo* mutagenicity tests. Dose selection for *in vivo* genotoxicity testing requires estimation of the maximum tolerated dose, consideration of tissue-specific effects and appropriate toxicokinetic data to support tissue exposure to the substances and/or metabolites.

66. The approach outlined to Stage 2 in figure 3 takes account of evidence to suggest that *in vivo* Comet and rodent transgenic mutation assays have better sensitivity and specificity for the identification of rodent carcinogens compared with the rat liver UDS test, particularly for carcinogens that are negative in the *in vivo* micronucleus test (Kirkland and Speit, 2008). The initial *in vivo* genotoxicity testing strategy should therefore involve selection of one or more of the core Stage 2 tests in rodents; namely, the transgenic gene mutation tests, micronucleus tests (accompanied by specific assays for aneuploidy if necessary) or comet DNA damage assays in rodents. It is acceptable to undertake one *in vivo* genotoxicity test to investigate a specific

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mutagenic end point identified from Stage 1 *in vitro* genotoxicity tests. In some instances there may be a need to investigate more than one end point before reaching a full conclusion on *in vivo* mutagenic potential.

67. Stage 2 *in vivo* genotoxicity tests should be undertaken for test substances that are positive in any of the *in vitro* Stage 1 genotoxicity tests where there is a need to ascertain whether genotoxic activity can be expressed *in vivo*. There are many reasons why activity shown *in vitro* may not be observed *in vivo* (for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination). Data from *in vivo* genotoxicity tests are, therefore, essential before any definite conclusions can be drawn regarding the potential mutagenic hazard to humans from test substances which have given positive results in one or more *in vitro* genotoxicity tests. However, conclusions on mutagenic hazard and MoGA may have to be derived from *in vitro* genotoxicity data for test substances when no *in vivo* genotoxicity testing is permitted.

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

69. Positive results in any Stage 2 genotoxicity test should be assessed for an indication of a MoGA and for evidence which may suggest threshold or irrelevant positive responses. Examples of such modes of action in micronucleus tests, for instance include compound induced by hypothermia or hyperthermia in rodents and compound induced increases in cell division of bone marrow erythroblasts (Tweats et al., 2007a, Blakey et al., 2008). If the conclusion is reached that a genotoxic mode(s) of action occurs then the chemical should be considered as an *in vivo* mutagen. MoGA data will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The COM has published guidance on possible threshold modes of genotoxicity which can include; i) involvement of non-

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Comment [.39]: Previous COM statement on high dose positives here?

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DNA targets, (e.g. aneugen inhibition of microtubules), ii) the contribution of protective mechanisms (e.g. repair of DNA adducts formed from many small molecular weight alkylating agents) and, iii) overload of detoxication pathways (e.g. paracetamol).

(http://www.iacom.org.uk/guidstate/documents/Thresholdsforinternetfinal.pdf)

70~~1~~. Supplementary *in vivo* tests should be undertaken if the results of the initial core *in vivo* genotoxicity tests give equivocal results or if there is a need to investigate specific mutagenic endpoints, tumour target organs, or the potential for heritable effects. This may involve repeating all or aspects of the initial Stage 2 testing strategy, or performing supplementary investigations (e.g. mode of action investigations, such as DNA adducts or more specific germ cell testing) to investigate aspects of the genotoxicity of the test substance which have not been resolved. There is a need to select the most appropriate test(s) on a case-by-case basis. All relevant factors, such as results from previous tests, and available information on toxicokinetics, toxicological effects and metabolism of the chemical, should be considered.

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71~~2~~. One aspect of the approach to testing outlined in Figure 2~~3~~ is that hazard characterisation of germ cell genotoxicity can be included in the initial *in vivo* genotoxicity testing strategy. This is because there are multi tissue *in vivo* genotoxicity assays (e.g. transgenic mutation assays~~ and comet assay~~) which can also be used if a need to evaluate germ cell genotoxicity has been established, although further evaluation of these assays for this purpose is recommended. Additionally, ~~a small number of ~~germ cell mutation assays might be valuable on a case-by-case basis to provide information on heritable mutagenic effects, but these would form part of a supplementary *in vivo* genotoxicity testing strategy, if considered appropriate.

72~~3~~. The COM reaffirms that a chemical considered a positive *in vivo* somatic cell mutagen should also be considered as a possible germ cell mutagen unless data can be provided to the contrary. The position held previously, that most if not all germ cell mutagens are also genotoxic in somatic cells still holds true. It has been noted that there are some rare examples (e.g. sodium orthovanadate, Attia et al., 2005) where the mouse bone marrow ~~ouse~~

micronucleus assay does not predict germ cell genotoxicity. However the data on such compounds are conflicting and it is not known, for example, whether somatic mutations would have been identified if other test systems (e.g. transgenic assays) had been used (Ciranni et al., 1995, Witt et al., 2003, Attia et al., 2005). It is possible these examples may relate to cellular targets in germ cells that are not present in the bone marrow (e.g. different proteins in chromatin structure and processes involved in meiosis). However, induction of other genotoxic effects and in other tissues cannot be excluded. There are also examples of germ cell mutagens which affect specific stages of gametogenosis in males (Adler, 2008) and where there are differences between male and female germ cell genotoxicity (Bishop, 2003).

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

Discussion of Stage 2 Initial Testing Strategy- General Aspects

74. There are many recent publications debating *in vivo* genotoxicity testing strategies. For example, the GUM (German Speaking section of the European Environmental Mutagen Society) recommended a single study using a combined analysis for micronuclei and comet induction in selected tissues (Pfuhler et al., 2007), while the WHO/IPCS recommended cytogenetics (bone marrow) or gene mutation or alternative tests as defined by genotoxic end-point chemical class and reactivity (with consideration of factors such as bioavailability and metabolism) (Eastmond et al., 2009). The *in vivo* genotoxicity testing strategy recommended by the COM acknowledges there can be a variety of reasons for undertaking *in vivo* genotoxicity tests and it is important to identify clearly the critical aspects of *in vivo* genotoxicity to be addressed (as set out in the Overview of Stage 2 strategy) which have to be investigated in order to develop a strategy

accordingly, rather than simply specify preferred first and second tests. There are less data on the performance of *in vivo* genotoxicity assays for prediction of rodent carcinogenicity compared to data on the performance of *in vitro* genotoxicity tests. Transgenic rodent assays (TGR) and the *in vivo* micronucleus assay have been shown to exhibit significant complimentarity regarding prediction of rodent carcinogenicity, consistent with the assessment of different mutagenic end points by these two assays. ~~(Lambert et al., 2005).~~ TGR was usually positive for those carcinogens which were positive in *in vitro* gene mutation tests in bacteria whilst the *in vivo* MN assay had greater predictivity for carcinogens positive in the *in vitro* metaphase analysis in mammalian cells (Lambert et al., 2005). Thus genotoxic end-point and MoGA analysis of *in vitro* mutagenic activity is of considerable importance in helping to develop an initial *in vivo* genotoxicity testing strategy. The COM recommends that the initial *in vivo* genotoxicity testing strategy should be based on one or more tests selected from a relatively limited number of *in vivo* genotoxicity tests that have been specifically designed to provide the optimum amount of information on *in vivo* mutagenic potential of the test substance.

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

75~~6~~. Three recommended *in vivo* genotoxicity tests are outlined below and in Figure 2~~3~~. Information from one or more of these recommended core tests should provide sufficient *in vivo* genotoxicity data for most chemicals.

~~*Rodent Bone Marrow MN and CA Assays for Clastogenicity and Aneuploidy*~~*Rodent Bone Marrow and Peripheral Blood MN Assay for Clastogenicity and Aneuploidy*

Rodent Bone Marrow CA Assay for Clastogenicity

76~~7~~. The *in vivo* bone marrow micronucleus assay is still the most widely used *in vivo* genotoxicity test. Most of the available *in vivo* data on the mutagenicity of chemicals have been obtained from studies using the bone marrow micronucleus assay (BMMN) in mice. The bone marrow is readily accessible

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to chemicals that are present in the blood and a wide range of structurally diverse clastogens and aneugens has been detected using these methods. The BMMN assay detects clastogenicity by measuring micronuclei (MN) formed from acentric chromosome fragments in young (polychromatic) erythrocytes in the bone marrow (or reticulocytes of peripheral blood). It may also be used to identify the induction of numerical aberrations. Micronuclei containing whole chromosomes (as opposed to fragments) can be identified with molecular kinetochore or centromeric labelling techniques. It should be noted that only aneuploidy produced by chromosome loss can be measured in the BMMN assay. The use of peripheral blood is an alternative approach for both mice (CSGMT, 1995) and rats (when the youngest fraction of reticulocytes are sampled) which provides equivalent data to the bone marrow assay and is technically less demanding (Wakata et al., 1998, Torous et al., 2000, Suzuki et al., 2005a, Rothfuss, 2011)~~(Wakata et al., 1998, Torous et al., 2000, Suzuki et al., 2005a)~~. High throughput approaches to the peripheral blood MN assay have been published (Torous et al., 2000, De Boeck et al., 2005). The rodent micronucleus assay can be used in the initial *in vivo* genotoxicity strategy for generic testing for *in vivo* mutagenic potential and for assessment of clastogenicity and aneuploidy. Clastogenicity may be measured by metaphase analysis of chromosomal aberrations (CA) in bone marrow of rodents as an alternative approach to the use of the micronucleus assay.

77~~8~~. Proposals have been published to incorporate MN micronucleus assays into routine rodent 28 day subacute toxicity studies which have demonstrated the feasibility of such an approach (Krishna et al., 1998, Hamada et al., 2001, Madrigal-Bujaidar et al., 2008)~~(Kirshna et al., 1998, Hamada et al., 2001, Madrigal-Bujaidar et al., 2008)~~. The development of a simultaneous liver and peripheral blood micronucleus assay in young rats has also been reported (Suzuki et al., 2005b). The evidence from one evaluation of micronucleus tests conducted on samples from short-term, subchronic and from a few chronic studies in mice has been published. MN in polychromatic erythrocytes represent DNA damage occurring in the last 72h, whilst MN in normochromatic erythrocytes represent~~ed~~ average damage during the 30 day period prior to sampling (Witt et al., 2000).

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Transgenic Rodent Mutation (TGR) Assay for Gene Mutations

78~~9~~. There has been a significant increase in the number of studies undertaken with transgenic rodent mutation (TGR) assays published since the COM guidance published in 2000. These have been comprehensively reviewed (Lambert et al., 2005). There are sufficient data to assess the performance of the Muta™mouse, BigBlue® mouse and rat (including use of λ cII transgene), *LacZ*plasmid mouse, and the *gpt* delta mouse models. The TGR assay can be used to assess gene mutations in a wide range of rodent tissues (including germ cells) using all routes of administration (Lambert et al., 2005) and is particularly valuable when investigating gene mutation as the genotoxic endpoint~~MoGA~~. TGR assays have been reported to produce data that are generally compatible with the mouse specific locus test for germ line mutagens (Singer et al., 2006a). In addition TGR assays can be particularly useful for *in vivo* site-of-contact mutagen assessment (Dean et al., 1999). Guidance on appropriate approaches to protocol development has been published by the IWGT (Thybaud et al., 2003). Molecular analysis of induced mutations in transgenic targets can aid in interpretation of study results (particularly equivocal responses) and also provide mechanistic information. Further information particularly on non-carcinogens is required to assess the overall performance of TGR assays although the available data suggests best positive and negative predictivity was obtained using results from *in vitro* Salmonella mutagenicity tests and *in vivo* TGR assays (Lambert et al., 2005). There is a need to consider and validate the optimal protocol when using transgenic mutation tests with tissues with a slow turnover. The OECD published a Detailed Review Paper (DRP) on Transgenic Rodent (TGR) Gene Mutation Assays in 2009 and recommended the development of an OECD guideline (OECD, 2009). A draft OECD guideline was adopted in April 2011 ~~is currently under consideration~~ (OECD, 2010).

Rodent Comet Assay for DNA Damage

7980. The *in vivo* Ceomet assay detects a wide spectrum of DNA damage including repairable DNA damage. An overview of the types of genetic lesions detected is given above in paragraph 60. The *in vivo* Ceomet assay can detect substances that induce gene mutations and has produced positive results for nearly 90% of rodent carcinogens not detected by the rodent bone marrow MN assay (Kirkland and Speit, 2008). There have been significant developments with regard to the conduct of the *in vivo* alkaline Ceomet assay since 2000 (Hartmann et al., 2003, Brendler-Schwaab et al., 2005, Burlinson et al., 2007). This assay can be used for elucidating positive *in vitro* genotoxicity findings and to evaluate genotoxicity in target organs of toxicity (Hartmann et al., 2004) however, it would not be an appropriate follow-up for a substance demonstrating an aneuploidy mode of action based on *in vitro* findings. There is now consensus agreement on a protocol for most tissues which would be consistent with an OECD guideline (Burlinson et al., 2007). The cometComet assay can be used in a wide range of species with many tissues including agerm cells and can be applied to site-of-contact tests. In the absence of data indicating particular tissues of interest (e.g. toxic findings or tissue accumulation seen in other studies), comet analysis of the stomach/duodenum (to detect site of contact effects), and liver (to detect genotoxic metabolites) should be studied. The Committee considers that the *in vivo* cometComet assay has appropriate sensitivity to detect chemicals which induce both gene mutations and/or clastogenicity. With regard to the assessment of germ cell genotoxicity, measurement of DNA effects by the cometComet assay in sperm requires additional steps for chromatin decondensation. A protocol for standardisation of the germ cell cometComet assay has not yet been achieved (Speit et al., 2009). Thus the *in vivo* cometComet assay can be used as a core test in the initial *in vivo* genotoxicity testing strategy to assess DNA damage in multiple tissues in a single study and it is possible to include the cometComet assay within standard regulatory toxicity tests (Rothfuss et al., 2010) or within other *in vivo* genotoxicity tests (Vasquez, 2010) .

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

8~~1~~0. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver (Kennelly et al., 1993). The endpoint measured is indicative of DNA damage and subsequent repair in liver cells. The COM consideration of this assay and published evaluations suggest it gives broadly similar results to the *in vivo* ~~comet~~Comet assay with regard to identification of genotoxicity in the liver (http://www.iacom.org.uk/statements/UDS.htm). An analysis of the prediction of rodent carcinogens not identified by the micronucleus tests indicated that the ~~comet~~Comet assay was considerably better than the rat liver UDS assay at identifying rodent carcinogens (Kirkland and Speit, 2008). The COM's preference is to use the ~~comet~~Comet assay rather than rodent liver UDS in order to assess *in vivo* potential for DNA damage.

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Discussion of Stage 2-Supplementary Tests.

81~~2~~. Supplementary *in vivo* genotoxicity tests need to be considered on a case-by-case basis taking into account all relevant information. It is considered that for most chemicals, supplementary *in vivo* genotoxicity data should be unnecessary but on a case-by-case basis specific aspects of MoGA (e.g. nature of DNA adducts) and further characterisation of germ cell genotoxicity (e.g. characterisation of male and/or female germ cell clastogenicity including use of FISH, and the evaluation of heritable effects) may be required. DNA adduct studies can provide valuable information on potential genotoxicity as a follow up for *in vitro* mutagens which have yielded negative results in *in vivo* genotoxicity assays (Phillips et al., 2000). DNA adduct data (including type of adduct, frequency, persistence, repair process) can be used to inform on MoGA and its relationship to carcinogenesis, and should be considered in conjunction with other relevant data such as dosimetry, toxicity, genotoxicity and tumour data (Jarabek et al., 2009).

Comment [.44]: SFTG EEMS use of DNA adduct data has not been fully described WP DISAGREE

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82~~3~~. A brief outline of these additional Stage 2 methods is given in Table 1. Reference is also made in Table 1 to a number of tests for heritable genotoxic effects but it is noted that these tests which involve the use of many animals and demand a high level of expertise are comparatively rarely used. The COM is aware that there is the possibility that gender differences in germ cell

mutagenesis may exist and this aspect may need to be considered on a case-by-case basis (Eichenlaub-Ritter et al., 2007)~~(Eichenlaub-Ritte et al., 2007)~~.

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Table 1. Supplementary *in vivo* genotoxicity tests.

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA Adducts				
^{32}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 ^{32}P. (Phillips et al., 2000)
Covalent binding to DNA A variety of methods can be used such as those involving radioactive delay or isotopic measurements. (eg. ^{14}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)~~(Himmelstein et al., 2009;)~~
Supplementary investigations of germ cell mutagenicity				
Analysis for clastogenicity/ aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes	OECD	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	None available	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~~Comet assay	Double strand breaks and/or apurinic sites in sperm head DNA	None available	Provides information on genetic instability in sperm	(Trivedi et al., 2010)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes	EPA	Provides information on induction of DNA lesions	(Sotomajor and Sega, 2000)

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

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

1) Investigation of mutagenic end point(s) identified in Stage 1,

2) Investigation of genotoxicity in tumour target tissue(s),

3) Investigation of potential for germ cell genotoxicity,

4) Investigation of *in vivo* mutagenicity for chemicals where there is high or moderate and prolonged exposure.

5) Investigation of genotoxicity in site of contact tissues.

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

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

Possible Future Developments

84~~5~~. The COM is aware that new assays and toxicogenomic approaches are under development which might be of value within genotoxicity testing. These include the detection of gene mutations at the endogenous phosphatidylinositol glycan complementation group A gene (*Pig-A*), a reporter gene in peripheral red blood cells of mammals (Bryce et al., 2008b, Miura et al., 2009). This assay has potential advantage of integration into regulatory toxicity tests (Dertinger et al., 2010) and it is noted that *Pig-A* mutations increase with duration of dosing (Miura et al., 2009). Other ~~a~~potential tests include ~~nd~~ investigation of instability in expanded simple tandem repeats in male gametes and offspring to evaluate heritable mutations (Singer et al., 2006b). The development of new high throughput assays for the assessment of germ line mutations and the quantification of risk from such data may provide opportunities to protect future generations from mutated DNA sequences. Developments within the field of toxicogenomics are also likely to provide new methods for investigating ~~dentifying~~ genotoxic mechanisms and informing on MoGA. The COM have reviewed data generated in this field several times during 2008 and 2009 up to the drafting of this guidance statement but currently conclude that the evidence does not support the routine use of toxicogenomic approaches to genotoxicity testing (http://www.iacom.org.uk/papers/index.htm). A recent workshop held by the ILSI-HESI (Health Effects Institute) IVGT (In-Vitro Genetic Toxicity Testing) Project Committee reviewed 16 assays/technologies which were at various stages of development (defined as emerging to mature). The workshop highlighted emerging approaches to genotoxicity testing such as Enzyme-DNA films and DNA adductome studies which should be considered as part of the COM's remit to horizon scan for new developments (Lynch et al., 2010)

Comment [.46]: Littron thinks this requires further information or at least updating of references. DONE!

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Annex 1: |QSAR and Sensitivity and Specificity Data Considered by the COM.

1. Data for sensitivity (correct identification of rodent carcinogens) and specificity (correct prediction of non-carcinogens as assessed in rodent carcinogenicity bioassays) have been obtained from a number of publications. Information is available for SAR approaches, screening tests and genotoxicity assays (both individually and in combinations).

2. The figures quoted depend on the carcinogenicity data set used (e.g. Gold Carcinogenicity Potency database (http://potency.berkeley.edu/), the classification of genotoxicity test results (i.e. positive, negative, equivocal based on study authors results or subjected to independent peer review) and whether equivocal and/or technically compromised (inadequate) test results have been included in the analyses. Sensitivity/specificity data for genotoxicity tests using a sub-set of genotoxic carcinogens have not been published, because this would require considerable work to evaluate the mode of action for carcinogenicity for a large number of chemicals. Specificity data for identification of chemicals with no *in vivo* genotoxic activity (non-genotoxins) have not been published, as there are no published databases for such chemicals.

3. The Sensitivity and specificity data that have been reviewed by the COM are tabulated below (rounded to whole numbers)

Structure Activity Assessments

4. An example of a ruled based approach is DEREK (Deductive Estimation of Risk from Existing Knowledge). This was developed by Lhasa Ltd, (https://www.lhasalimited.org/derek/) (Marchant and Group, 1996) The output from statistically based models is a quantitative probability for the endpoint under consideration. Examples include MultiCASE (Multiple Computer Automated Structure Evaluation; http://www.multicase.com/) and TOPKAT (Toxicity Prediction by Komputer Assisted Technology; http://accelrys.com/mini/toxicology/predictive-functionality.html). Some databases and models for prediction of *in vitro* mutagenicity (including bacterial and mammalian cell systems) have been developed by the European

Chemicals Bureau (ECB) (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=QRF) and the US Food and Drug Administration (http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092221.htm).

5. The European Commission is funding the SCARLET (Structure-activity relationships leading experts in mutagenicity and carcinogenicity) project (www.scarlet-project.eu/). A model for mutagenicity prediction (e.g CAESAR (Computer Assisted Evaluation of industrial chemical Substances According to Regulations) is freely available (www.caser-project.eu) (Benfenati et al., 2009a)(Benfenati et al., 2009). A structural alert database for mutagenicity (Toxtree) is also freely available from the ECB internet site (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE) (Benigni and Bossa, 2008). The OECD Toolbox includes software and data bases for use in the prediction of genotoxicity and is also freely available. (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 and the appropriate documentation for demonstrating model validity (the QSAR Model Reporting Format (QMRF)) has been published by the European Chemicals Agency (Guidance on Information Requirements and Chemical Safety Assessment Chapter R6 http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm). Information on completed QMRFs for (Q)SAR models can be accessed at http://qsardb.jrc.it/qmrf/.

6. An authoritative and comprehensive evaluation of the different (Q)SAR approaches to the identification of genotoxic potential has been prepared for European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/en/scdocs/scdoc/50e.htm) by the Computational Toxicology group, Institute for Health & Consumer Protection, European Commission-Joint Research Centre (JRC), Ispra, Italy. A dataset comprising pesticides, the Distributed Structure-Searchable Toxicity (DSST) database and EU classified mutagens was used to assess a wide range of computer based predictive models. Overall the JRC concluded the (Q)SAR approaches reviewed were shown to produce acceptable results for the prediction of bacterial mutagenicity and that the use of a two-software combination

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(including assessment of (Q)SAR data on structural analogues of the chemical under consideration) can reduce the false negative rate for the identification of classified mutagens. A combination of CAESAR and Toxtree yielded a false negative prediction rate of 11% for classified mutagens.

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

Annex 1 Table 1 QSAR data

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	Agreement with Ames positive 65% (416 compounds) (Cariello et al., 2002)
TOPKAT	No data reported	No data reported	Agreement 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 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)

Annex 1 Table 2 Screening Tests

Method	Sensitivity[a] Identification of mutagens	Specificity[b] 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%) 95% for genotoxic carcinogens 63% (regulatory battery of Ames, CA/MNvit, or CA/MLA) 94% of genotoxicants Validation data for 56 compounds requiring metabolic activation 30% (in vitro genotoxicants) 30% (rodent carcinogens)	22/23 ECVAM list of mammalian cell non-mutagens (96%) 100% non-carcinogens 100% (regulatory battery of Ames, CA/MNT or CA/MLA) 83% non-genotoxicants 97% (in vitro genotoxicants) 88% (rodent carcinogens)	(Birrell et al., 2010) 75 compounds studied (Hastwell et al., 2006) Validation data for 56 compounds requiring metabolic activation (Jagger et al., 2008) 57 *in vitro* genotoxicants ns 50 chemicals with rodent carcinogenicity data. (Olaharski et al., 2009)

a: accurate prediction of positive responses compared to comparator dataset, given in parenthesis

b: accurate prediction of negative responses compared to comparator dataset, given in parenthesis

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Annex 1 T~~t~~able 3 Genotoxicity Tests (*in vitro*) in Relation to Rodent Carcinogenicity

Method	Sensitivity [a]	Specificity [b]	Comments/references
Ames	59%	74%	541 chemicals (Kirkland et al., 2005a)
Ames	52%	72%	3711 chemicals including tests with *Salmonella* and *Escherichia* (Mathews et al., 2006)~~(Matthews et al., 2006)~~
Micronucleus (*in vitro*)	79%	31%	89 chemicals (Kirkland et al., 2005a)
Micronucleus (*in vitro*)	88%	23%	182 chemicals (Mathews et al., 2006)~~(Matthews et al., 2006)~~
Chromosomal aberrations (*in vitro*)	66%	45%	352 chemicals (Kirkland et al., 2005a)
Chromosomal aberrations (*in vitro*)	55%	63%	1391 chemicals (Mathews et al., 2006)~~(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, Mathews et al., 2006)~~(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)

*Positive results in at least one test

a: accurate prediction of rodent carcinogenicity

b: accurate prediction of rodent non-carcinogenicity

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

5 1.

Chemicals evaluated	Sensitivity[a] Ames +MN*	Sensitivity[a] 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 fail to be detected ~~be 'missed'~~ by Ames + MN.
409 *in vivo* genotoxins	(78%) 317/409 Remainder were negative, but technically compromised, weak, equivocal or inconclusive/insufficient detail.	(79%) 323/409 4/6 of the additional *in vivo* genotoxins detected by MLA had not been tested in either MN or CA.	No convincing evidence that any *in vivo* genotoxins would fail to be detected ~~be 'missed'~~ by Ames + MN.

6
7 a: accurate prediction of either rodent carcinogenicity or *in vivo* genotoxicity (see chemicals evaluated
8 column)

9 * ~~or~~ chromosomal aberration data where no micronucleus test was available. Abbreviations MN=
10 micronucleus test, MLA = mouse lymphoma assay, CA= chromosomal aberration assay

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Annex 2. Tabulation of Genotoxicity Tests (in Stages 1 and 2) and 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 *S. typhimurium* tester strains and *E. coli* ~~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
~~Chromsomal~~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, aneuploidy	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~~Comet assay	DNA strand breaks, alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action.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	E~~e~~ndpoint measured is indicative of DNA damage and subsequent repair in liver cells. ~~Broadly similar response compared with comet assay.~~

Annex 3:

Rationale for Selection of Ames Test and *In Vitro* Micronucleus Assay as The Two Principle In *Vitro* Assays.~~(Kirkland Et Al., 2010)~~ (Kirkland et al., 2011)

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

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

A. Toluene was reported positive in the NTP MLA study, but has subsequently been re-evaluated as equivocal in the analysis of (Schisler et al., 2010), and was not found

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positive in a rigorous MLA conducted to higher concentrations and >80% toxicity (Kirkland and Fowler, 2010).

B. Benzyl acetate was reported positive in the NTP MLA study, and subsequently re-evaluated as positive by Schisler et al (2010), but an expert panel review (Mitchell et al, 1997) identified this chemical as "untestable" in the MLA because it reacts with the plastic of the culture vessels and may thus produce artefacts.

C. Morphine was negative in a non-standard *in vitro* micronucleus test in which mouse splenocytes were treated only for 21 hr in the absence of metabolic activation. It is possible that morphine may induce micronuclei when tested at higher concentrations over shorter periods in the absence and presence of metabolic activation in a standard assay.

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D. Thiabendazole is an aneugen which, typically, has a very steep dose response. It has been found positive for induction of micronuclei *in vitro* in several papers, but is reported equivocal or negative in other papers, possibly because optimum concentration spacing, treatment and sampling times were not used.

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

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4. The revised strategy of two tests (Ames and MNvit) allows for the efficient identification of all mutagenic end points but, by reducing the number of mammalian cell tests from that recommended by COM in 2000, and following improved methodologies, the risk of misleading positive results is decreased.

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GLOSSARY

A glossary of terms used in COT reports is available which provides useful information on may of the terms cited in this COM guidance document. http://cot.food.gov.uk/moreinfo/cotglossary A glossary of additional terms used in this guidance document is given below.

Additional terms

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

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

Erythrocyte: red blood cell; corpuscle; one of the formed cells in peripheral blood. Normally, in humans, the mature form is a non-nucleated, yellowish, biconcave disk, containing haemoglobin and transporting oxygen.
Normochromic erythrocyte; one of normal colour with a normal concentration of haemoglobin.Polychromatic erythrocyte; one that, on staining, shows shades of blue combined with tinges of pink indicative of an immature erythrocyte.

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

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

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

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

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

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

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

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

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

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

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

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

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 od DNA structure with balanced or unbalance 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/β-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 which has the capacity to metabolise chemicals *in vitro*.

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

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