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Annex 1 to MUT/2010/09~~1~~

GUIDANCE ON A STRATEGY FOR TESTING OF CHEMICALS FOR GENOTOXICITY (2nd DRAFT)

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I. Preface

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) is an expert advisory committee whose members are appointed by the Chief Medical Officer for England and the Chair of the Food Standards Agency 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 committee is to advise all U.K. government departments and agencies with an interest in the safety of chemicals across various sectors, on all aspects of the mutagenicity and genotoxicity of chemicals. (These terms are defined for the purposes of this guidance document in paragraphs 7-9 below.) The Secretariat is provided by the Health Protection Agency (who lead) and the Food Standards Agency (FSA). Other government departments with an interest provide assessors to the Committee; these are specifically from the Department of Health, the Department of Environment, Food and Rural Affairs (D~~d~~efra), the Chemicals Regulation~~ory~~ Directorate (CRD) of the Health and Safety Executive (HSE) (responsible for legislation regulating chemicals, pesticides, biocides and detergents),~~approval of pesticides and biocides),~~ the Veterinary Medicines Directorate (VMD: a D~~d~~efra agency responsible for the licensing of veterinary drugs) and the Medicines and Healthcare 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 an agency that does have such a role (e.g. HSE CRD for occupational aspects and for pesticides etc). Its remit is to advise on all aspects of mutagenicity and genotoxicity of chemicals, and this may involve advice on a specific chemical, and also on testing strategies and research. The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with mutagenic and genotoxic hazards (the inherent property of the substance) or risk (the likelihood of mutagenic or genotoxic effects occurring after a given exposure) and to present recommendations for genotoxicity testing. In practice the bulk of the work of the Committee relates to assessing genotoxicity tests and providing advice on mutagenic hazard of chemicals.

4. In the context of testing strategies the COM first published guidelines for the testing of chemicals for mutagenicity in 1981 which were revised in 1989 (DOH., 1989). These provided guidance to the relevant government departments and agencies on best practice for testing at that time. The need for guidance to be periodically updated, to reflect advances in development and validation of methods, was recognised and revised guidance was published in 2000 (DOH., 2000). This new guidance continues this updating process. The strategy outlined is believed to be the most scientifically appropriate given available methods and recognises the need to avoid use of live animals where practical and where validated alternative methods are~~were~~ available. It is recognised that, as with the earlier published COM guidance, it will be some time before this strategy is reflected in ~~the mandatory, regulatory~~ guidelines used by UK regulatory authorities~~-~~. The COM guidance ~~of the various agencies, and it~~ is not intended ~~for the COM guidance~~ to be applied retrospectively for the genotoxicity testing of new substances. However specific guidance is given below for applying the COM testing strategy to the mutagenicity assessment of chemicals which have existing, and in many cases inadequate or incomplete genotoxicity data.~~.~~

5. The Committee believes that the approach outlined presents an overview of the ~~n~~core principles of genotoxicity testing and ~~here~~ will remain valid for

several years. It is hoped the revised COM guidance and will be well received both nationally and internationally and will encourage further debate regarding the principles of genotoxicity testing. The guidance is not intended to supersede or replace existing national or internationally 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) (ICHhttp://www.ich.org/cache/compo/276-254-1.html) and for chemicals assessed under (REACH) (http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm). encourage international recognition of the newer assays being recommended for which there are, currently, no internationally harmonised guidelines.

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II. Introduction

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

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Details of methodologies are not given since they are provided in the OECD test guidelines and IWGT guidance.

7. Genotoxic (or genotoxicity) refers to agents that~~which~~ 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 includes mutation as well as 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. However the detection of such effects does not in itself provide direct evidence of inherited mutations. The term "genotoxic carcinogen" as used by the Committee described those chemicals that have been demonstrated to be carcinogenic in humans and animals and are considered to be *in vivo* mutagens. This guidance presents a strategy for genotoxicity testing since this term encompasses all the assays included in the strategy. The COM evaluates the results of the available tests on a particular substance and provides advice on mutagenic hazard.

8. ~~8.~~ In this guidance document the term mutation refers to 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. 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. Changes involving chromosomes as entities may be numerical or structural. It is important to generate information on the three levels of mutation, namely gene, clastogenicity (i.e. structural chromosome changes) and aneuploidy (i.e. numerical chromosomal changes), to provide comprehensive coverage of the mutagenic potential of a chemical.

9. The Committee reaffirms its view published in 1989 and 2000 that there is currently no single validated assay that can provide comprehensive information on all three end-points, namely gene mutation, clastogenicity and

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aneuploidy and thus it is necessary to subject a given substance to several different assays. A range of assays has been developed which employs a wide variety of organisms, including prokaryotes (bacteria), yeasts and other eukaryotic microorganisms, and mammalian cells studied *in vitro*, as well as whole mammals where effects in a wide range of target organs including germ cells can be measured. A number of different end-points can be used which may measure genetic changes or indicators for the potential to produce genetic change. Assays may be classified on the basis of genetic endpoints (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.

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III Significance of chemical induced mutation for human health

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10. 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 descendent 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.

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Evidence for chemical inherited genetic changes

11. A full description of the impact of mutation on human health and classification of inherited defects is beyond the scope of this guidance and the reader is referred to published reference text books.(Turnpenny and Ellard, 2005, Lynch, 2009) In brief, there are many thousands of monogenic (single gene) genetic disorders which have been identified. Detailed information can be found in the online Mendelian Inheritance in man database (www.ncbi.nlm.nih.gov). There is no convincing evidence for chemical induced inheritable germ cell mutations in human populations (Lynch, 2009), although developments in molecular epidemiology may help to address the need for improved study designs (Elespuru and Sankaranarayanan, 2007).

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12. There is evidence that exposure of women during pregnancy to *in vivo* mutagens (e.g. anti cancer drugs such as busulfin, cyclophosphamide, cytarabin, 6-mercaptopurine and daunorubicin/doxorubicin) can lead to transplacental exposure of the fetus with subsequent teratogenic effects.(Bishop et al., 1997). There is also convincing evidence from studies using experimental animals that *in vivo* mutagens can cross the placental barrier and increase mutation frequency in the fetus. Examples include ethyl nitrosourea (Mei et al., 2005), cisplatin (Munoz et al., 1996) urethane (Nomura, 2008) and polycylic aromatic hydrocarbons such as 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene and benzo(a)pyrene (Donovan et al., 2004). In addition biomonitoring for DNA damage (using the comet assay) in peripheral blood lymphocytes sampled from umbilical cord blood taken from newborn infants shows significantly higher DNA damage in infants whose mothers were active smokers compared to infants born to mothers who did not smoke (de Assis et al., 2009).

13. There is less evidence for paternal exposure to *in vivo* mutagens resulting in inherited genetic changes. Studies of sperm from smokers show evidence for increased levels of oxidative DNA damage and benzo(a)pyrene diol epoxide DNAaducts -and evidence that these adducts are transmitted to embryos (Chang, 2008). A number of epidemiological studies have documented evidence for an association between paternal smoking and childhood leukaemia (Chang, 2008). However overall the evidence for an association between paternal smoking and childhood leukaemia is limited (Wigle et al., 2008)

Mutagenic effects in carcinogenesis.

14. -DNA damage in a somatic cells may result in a somatic mutation, which may lead to malignant transformation (cancer). A substantial proportion of the known human carcinogens exhibit *in vivo* mutagenic activity (http://monographs.iarc.fr/ENG/Classification/crthalllist.php). A mutagenic mode of action (MOA) for carcinogenesis has been reported for a number of these genotoxic carcinogens where a detailed assessment has been published (e.g. cyclophosphamide(McCarroll et al., 2008) and chromium (IV) (McCarroll et al., 2010)). Characterisation of gene mutations in human

tumours, in common with the known mutagenic profiles of genotoxins in experimental systems, may provide further insight into the role of environmental mutagens in human cancer (Phillips and Arlt, 2009). Mutational inactivation of tumour suppressor genes and activation of oncogenes are associated with development of a wide range of cancers. (Dixon and Kopras, 2004).

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9. It is important to generate information on the three levels of mutation, namely gene, clastogenicity (i.e. structural chromosome changes) and aneuploidy (i.e. numerical chromosomal changes), to provide comprehensive coverage of the mutagenic potential of a chemical. This is also the case when assessing carcinogenic potential, since all three types of mutation have been shown to be associated with the activation and expression of oncogenes, and loss or inactivation of tumour suppressor genes and other classes of genes implicated in carcinogenesis.

10. The Committee reaffirms its view published in 1989 and 2000 that there is currently no single validated assay that can provide information on all three end-points, namely gene mutation, clastogenicity and aneuploidy and thus it is necessary to subject a given substance to several different assays. A range of assays has been developed which employs a wide variety of organisms, including bacteria, yeasts and other eukaryotic microorganisms, and mammalian cells studied *in vitro*, as well as whole mammals where effects in a wide range of target organs including germ cells can be measured. A number of different end-points can be used which may measure genetic changes or indicators for the potential to produce genetic change. Assays may be classified on the basis of genetic endpoints (e.g. gene mutation, clastogenicity, aneugenicity and tests for DNA damage) or by consideration of the different phylogenetic levels represented and also in mammals by the tissues or target organs studied.

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IV~~II~~. General principles of testing strategy

15. The Committee recommends a two-stage genotoxicity testing strategy for the detection of mutagenic hazard of new and existing chemicals which can be supported by appropriate pre-screening and/or *in silico*~~for the detection of mutagenic hazard~~ data (Stage 0). A case-by-case testing strategy should be developed for chemicals which have existing inadequate and/or incomplete genotoxicity data. ~~.~~ Initial screening for mutagenic activity in Stage 1 is based upon three [or two~~ in those cases where little or no human exposure is expected e.g. industrial intermediates, some low product volume chemicals~~] *in vitro* tests with case-by-case additional testing and investigation depending on the results of the initial screening tests. The same approach to initial screening is used for chemicals where ~~no~~ *in vivo* genotoxicity testing is not permitted~~included in regulatory testing strategies~~ (e.g. cosmetics). Investigations regarding mode of action (MOA) are important to derive conclusions on biological significance of genotoxicity tests 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. Stage 2 consists of a number of *in vivo* tests designed to investigate whether *in vitro* mutagenic 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. ~~where information on *in vivo* mutagenic potential is required.~~ There is currently no single *in vivo* test which can assay all three genetic endpoints (Thybaud et al., 2007)~~(Thybauld et al., 2007)~~ and thus a strategy for S~~s~~tage 2 has to be designed based on the nature of genotoxic ~~mutagenic~~ effects identified in Stage 1.

16.

~~12.~~There should be a~~is a~~ clear strategy for planning tests within each stage and for progressing to Stage 2 (see Figs 1 and 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 developing a strategy for S~~s~~tage 2 is more complex and needs to be developed on a case-by-case basis.

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~~13.~~17. All *in vitro* assays should be designed to provide the best chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system; (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 ~~mutational~~ event. The assays should be carried out as far as is possible to the internationally recognised guidance and protocols (e.g. as published by the Organisation for Economic Cooperation and Development (OECD), and the International Working Group on Genotoxicity Testing (IWGT))~~.~~

~~14.~~18. Few chemicals are active only *in vivo* and in such cases this may be due to~~for~~ a number of reasons 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).

19~~8.~~ Under the strategy recommended by the Committee, the use of animals in mutagenicity testing is primarily required when it is necessary to investigate whether genotoxic ~~mutagenic~~ activity detected *in vitro* is reproduced *in vivo,* target organ genotoxicity (for example involvement of genotoxicity in rodent tumours) and potential for heritable mutagenic effects. Genotoxicity testing using animals should be carried out judiciously and sparingly taking into account factors such as the results of Stage 1 tests, evidence for tumours identified in rodent carcinogenicity tests(Kirkland et al., 2007c), and the likelihood of high, or moderate and prolonged human exposure.~~Except in those cases where high, or moderate and prolonged human exposure is expected, (e.g. many human medicines) or in some instances where tumours are subsequently identified in rodents,(Kirkland et al., 2007c) there is no justification for the routine use of animals for mutagenicity tests when there is no evidence for activity at Stage 1~~.

Additional considerations for chemicals with limited and/or inadequate genotoxicity data

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2019. The principles for a genotoxicity testing strategy for chemicals with limited and/or inadequate genotoxicity data are essentially similar to those required for a new substance and are based on Stages 0, 1 and 2 outlined above, although a step wise approach to a genotoxicity testing strategy needs to be taken on a case-by case basis using the following steps;

a) Consider the purpose of the testing strategy using the following objectives. Is test strategy designed to 1) Screen for *in vitro* and/or *in vivo* mutagenic potential. 2) Investigate genotoxicity in tumour target tissue(s), 3) Investigate potential for germ cell genotoxicity, 4) Investigate mutagenic end point(s) identified in existing genotoxicity data.

b) Assess individual studies for which genotoxicity data are available and reach conclusions on whether each study is adequately or inadequately conducted and reach conclusions on genotoxicity data (i.e. positive, negative or equivocal). Use appropriate criteria to decide if any positive or equivocal result may be misleading and determine MOA. Use available SAR approaches as an additional aid in the evaluation of genotoxicity tests. Such SAR data may also be helpful in identifying misleading negative genotoxicity tests results.

c) Attribute weight of evidence to each genotoxicity data point. In general the highest weight of evidence should be attributed to the core studies outlined in the COM strategy with less weight of evidence for other studies.

d) From weight of evidence genotoxicity evaluation and conclusions reached on individual genotoxicity tests consider if conclusions can be derived for each stage of the COM testing strategy (i.e. Stages 0,1, and 2).

e) Identify key data gaps taking into account the purpose of the evaluation and derive a testing strategy for each stage of the COM testing strategy as appropriate. This may include repeating specific genotoxicity tests from each stage of the COM testing strategy and/or undertaking additional core studies from Stages 1 and 2 as appropriate. The extent of any testing strategy will primarily be aimed at bridging genotoxicity data

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gaps relevant to the purpose of the genotoxicity evaluation as set out in paragraph a) above and will depend on the priority for assessment and available resources.

210. If there are very few data available as a minimum useful information may be gleaned from publicly available Structure Activity Relationship (SAR) databases can provide helpful information to aid in deciding priorities for genotoxicity testing. The rationale published by Dearfield et al 2010 with regard to follow-up testing of positive *in vitro* mutagenic effects which involves interpreting data, assigning a weight of evidence to specific genotoxicity data, and reaching decisions on data gaps in order toderive conclusions on further testing is a valuable guide to aid in decision making using the approach given above for chemicals with existing limited and/or inadequate genotoxicity data.

15.

16. A short overview of the rationale supporting the approach recommended by the Committee is given below, along with some brief comments on matters to consider before devising a testing strategy for a specific test substance.

V Genotoxicity Testing Strategy for new and existing substances

IV Stage 0: Prescreening considerations prior to genotoxicity testing

17.20. The intrinsic chemical properties of the test substance must be considered before devising the genotoxicity mutagenicity testing programme.

Physico-chemical and toxicological properties

21. The physico-chemical properties of the test substance (for example, pH, solubility, and stability in solvents/vehicles) and its purity can affect the ease of conduct and results of *in vitro* tests. For example, the tolerance of

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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 confound the outcome of *in vitro* genotoxicity tests(Long et al., 2007). The toxic properties of test substances (such as acute toxicity, subchronic toxicity (including 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 *in-vivo* mutagenicity tests. Dose selection for *in-vivo* ~~t~~genotoxicity testing requires estimation of the maximum tolerated dose, consideration of tissue-specific effects and appropriate toxicokinetic~~pharmacokinetic~~ data to support tissue exposure to the substances and/or metabolites.

Structure Activity Relationships

~~19.~~22. Whether the test substance would be expected to have mutagenic potential can be assessed from its chemical structure and which may provide structural alerts for mutagenicity. A composite model structure was devised by Ashby and Paton in 1993 indicating substituent chemical groups or moieties associated with DNA-reactivity (Ashby and Paton, 1993). A number of published and commercial systems to investigate structure activity relationships (SAR) have been investigated (Zeiger et al., 1996, Cariello et al., 2002, Contrera et al., 2005, Snyder and Smith, 2005, Benigni et al., 2007, Benigni and Bossa, 2008). The OECD and the European Commission (Joint Research Centre) have published principles for the validation of (Q)SAR ((Quantitative) Structure Activity Relationships)(OECD., 2004, Worth et al., 2005, Benigni and Bossa, 2008). One approach is to predict *in vitro* mutagenicity in bacteria by automated analyses of the statistical correlation between structure and mutagenic activity and/or programmed rules for prediction based on the available knowledge and expert judgement. An example of a ruled based

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approach is DEREK (Deductive Estimation of Risk from Existing Knowledge).(Jacobson-Kram and Contrera, 2007) In contrast (Q)SAR softwares are statistically based programs andthat produce computer generated equations (models) relating to chemoinformatic information. The output is a quantitative probability of the endpoint under consideration. Examples include MultiCASE (Multiple Computer Automated Structure Evaluation) and TOPKAT (Toxicity Prediction by Komputer Assisted Technology)(Jacobson-Kram and Contrera, 2007). The FDA consensus modelling approach is to use (Q)SAR systems in conjunction with expert rule systems (Custer and Sweder, 2008). Some databases and models for prediction of *in vitro* bacterial mutagenicity (including bacterial and mammalian cell systems) have been developed for use by Regulatory Agencies by the European Chemicals Bureau (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=QRF and the US Food and Drugs Agency http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092221.htm The European Commission is funding the SCARLET (Structure-activity relationships leading experts in mutagenicity and carcinogenicity) project. Models for mutagenicity prediction (e.g CAESER (Computer Assisted Evaluation of industrial chemical Substances According to Regulations) will be made freely available (www.caser-project.eu) (Benfenati et al., 2009). A structural alert database for mutatgenicity (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).

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20.23. Such systems can be useful when a large number of compounds require assessment and prioritisation for biological testing in bacteria. 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 reasonably well for prediction of mutagenicity in *Salmonella* (particularly within specific chemical classes included in the training set)(Matthews et al., 2006, Jacobson-Kram and Contrera, 2007, Benigni and Bossa, 2008, Benfenati et al., 2009.). The sensitivity and

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specificity of *Salmonella* bacterial mutagenicity prediction using the FDA MDL QSAR model was 81% and 76% respectively (Contrera et al., 2005). In general lower sensitivities and specificities have been reported for a number of systems when used for prediction of results from *in vitro* cytogenetics (using MCASE (Matthews et al., 2006, Roithfuss et al., 2006) (44%, 92% respectively) and MDL-QSAR (60.7%, 76.2% respectively (Contrera et al., 2008))) and the mouse lymphoma assays (MCASE (63%, 74% respectively) MDL-QSAR (73.8%, 63.0% respectively) (Contrera et al., 2008). One factor in the lower prediction of SAR systems for mammalian cell genotoxicity assays is inadequate coverage of non-covalent DNA interactions (Grant et al., 2000, Snyder and Smith, 2005). It has also been proposed that SAR assessments can aid in the interpretation of the relevance of *in vitro* genotoxicity assays through prediction of biotransformation (Combes et al., 2007).

24. 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 as a coarse-grain filter for preliminary screening of *in vivo* mutagens (Benigni et al., 2010).

25. Overall *in silico* approaches to prediction of genetic toxicology can be a valuable tool for high throughput screening new compounds (prioritisation for testing) and for aiding in the interpretation of genetic toxicology tests for both new and existing chemicals, although currently the data cannot replace the need to undertake appropriate *in vitro* and *in vivo* genotoxicology tests required to derive conclusions on mutagenic hazard.

Prescreening tests

~~21.~~26. There are a number of current initiatives which attempt to combine data mining *in silico* approaches with high throughput tests to develop approaches to pre-screening large numbers of novel chemicals (Benfenati et al., 2009.). Prescreening tests need to be rapid, economical, reproducible, requiring only small amounts of test substances (typically below 50 mg) and have a high concordance with comparator end points

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in full regulatory tests. Prescreening high through-put bacterial tests have been developed using primary DNA damage (*umu* assay), ~~mutations in histidine requirement (fluctuation test), and~~mutations in ampicillinase gene (MutaGen assay), bioluminescence or 5-fluorouracil resistance (Reifferscheid et al., 2005, Miller et al., 2005 , Aubrecht et al., 2007, Ackerman et al., 2009.). Other pre-screening systems cited in the literature include DNA repair activity in yeast cells(Westerink et al., 2009)~~(Westerlink et al., 2009)~~. One research group has proposed a combination of two commercial pre-screening assays (Vit~~i~~otox™ for bacterial mutagenicity and RadarScreen yeast screen for clastogenicity) for rapid screening of compounds and de-selection for genotoxicity (Westerink et al., 2009)~~(Westerlink et al., 2009)~~.

~~22.~~27. A number of pre-screening genotoxicity tests using mammalian cells have been proposed including oxidative reactions of adducted pyrimidine bases in calf thymus(Garas et al., 2009), alkaline elution using rat hepatocytes (Gealy et al., 2007), the detection of DNA damage (via p53 or GADD45a activation, Green Screen) in cell lines (Knight et al., 2009) and differential growth in DNA repair proficient and deficient cell lines(Helleday et al., 2001). A pre-screening assay 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 prescreening assays 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 pre-screening tests is as an aid in development prioritisation of compounds undertaken by industry. The Green Screen has been developed as a pre-screening tool with better concordance with carcinogenicity outcome ~~than~~ than some regulatory genotoxicity tests (Custer and Sweder, 2008, Knight et al., 2009).

23.28. Prescreening genotoxicity tests can be used in a tiered approach with *in vitro* genotoxicity tests during chemical development. One proposal for the future is that greater validation and acceptance by regulatory authorities of pre-screening tests might possibly lead to the replacement of existing genotoxicity testing strategies with a combination of high throughput screening tests (Custer and Sweder, 2008).

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Stage 1: Initial *in In vitro genotoxicity testingscreening*

Introduction: Overview of strategy

24.29. The strategy recommended in the following sections is concerned with investigating mutagenic activity of individual chemicals and no consideration is given in these guidelines to mixtures of chemicals. The Committee 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 false negative results. Since the publication of its guidelines in 2000, assessments of the performance of genotoxicity assays (both individually and in combinations) regarding the prediction of rodent carcinogenicity have been published (Kirkland et al., 2005a, Matthews et al., 2006, Kirkland and Speit, 2008). Reference to these publications can provide an insight into the performance of the genotoxicity assays for the data sets analysed regarding prediction of carcinogenicity/non carcinogenicity status, but it is noted that the database for some assays (e.g. *in vitro* micronucleus assay) is limited. 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 avoid misleading false positive results.

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25.30. As outlined above, Stage 1 involves screening tests for mutagenic activity using *in vitro* methods and comprises a three (two) test-system with the objective of assessing mutagenic potential for three end points (gene mutation, chromosomal damage and numerical changes in chromosome number). A clearly positive result in any one of the three (two) tests is sufficient to define the chemical as an *in vitro* mutagen.

Comment [j2]: This may alter to two tests depending on further consideration of utility of MLA.

The nature of mutagenic effects detected in Stage 1 should inform on the choice of tests identified for Stage 2 (which includes an initial and supplementary *in vivo* genotoxicity testing strategy). Additional investigations for chemicals which give positive or repeated equivocal results in Stage 1 tests can include assessment of mode(s) of *in vitro* genotoxic action (MOA). Misleading ~~false~~ positive results have been reported particularly for certain mammalian cell assays (Kirkland et al., 2007a, Pfuhler, 2009). There are a number of reasons (discussed below) why positive results in *in vitro* genotoxicity tests might occur by mode(s) of action not relevant to human health hazard assessment. It is particularly necessary to undertake a MOA evaluation for those chemicals (e.g. cosmetics) where there is a regulatory constraint which eliminates the use of *in vivo* genotoxicity assays in the testing strategy. It is necessary to obtain clearly negative results in all *in vitro* tests undertaken in order to reach a conclusion that the chemical has no mutagenic activity. Usually data from all three (two) tests in Stage 1 will be necessary. ~~but in the case of those substances where there will be little or no human exposure, (e.g. industrial intermediates and some low production volume chemicals) the mammalian cell mutation assay can be omitted.~~

~~26.~~31. There are some occasions where additional *in vitro* genotoxicity testing may be undertaken for chemicals giving a negative response in three (two) standard *in vitro* genotoxicity tests, for example where tumours are subsequently found in rodents and there is evidence that specific rodent or human metabolites need to be subject to genotoxicity assessment, or the test substance has a structural alert but standard *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 aspect (Kasper et al., 2007). Further information on *in vivo* genotoxicity testing of such test substances is provided in Stage 2 of this strategy. An important part of any additional *in vitro* strategy would be consideration of the appropriate exogenous metabolic activation system (including alternative

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sources of S-9, other metabolic systems including genetically engineered cell lines)(Ku et al., 2007b).

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27.32. For chemicals which give equivocal results or repeated low magnitude positive results it is important to consider evidence of reproducibility, and the magnitude of effect in relation to historical negative control data and then consider if further *in vitro* genotoxicity testing is warranted (Kirkland et al., 2007b). Further consideration of MOA and SAR data for these chemicals can also give valuable information (Dearfield et al., 2010).

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28.33. Additional tests using reconstructed human skin may be undertaken on a case-by case basis to provide information on chemicals which give equivocal or positive results in Stage 1 *in vitro* tests in circumstances where *in vivo* testing will not be performed (for example with cosmetic ingredients).

29.34. The full Stage 1 strategy should be performed and the results of studies evaluated before a decision is made as to whether to proceed to Stage 2 testing or for test substances where no *in vivo* genotoxicity testing is allowed to derive a conclusion on mutagenic hazard. An outline of Stage 1 (initial *in vitro* screening) is given in Figure 1 and a description of the assays recommended is provided in the following paragraphs.

Discussion of Stage 1 Tests

General aspects

30.35. The sensitivity of conduct of genotoxicity assays have improved over the years and overall sensitivity of *in vitro* testing strategies regarding prediction of rodent carcinogens is very high (Kirkland et al., 2007a). it is generally accepted that there is essentially a negligible chance of failure to detect genotoxic activity using the Stage 1 testing strategy (Kirkland et al., 2007a). It is most likely that the few occasions where this *in vitro* test strategies strategy fails to detect mutagenic activity will be due to the absence of appropriate metabolic activity *in vitro*. There have been published proposals for genotoxicity testing which either advocate a

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single *in vitro* genotoxicity test (Ku et al., 2007a) or a complex approach involving up to six *in vitro* genotoxicity tests (as reviewed by Kirkland and colleagues (Kirkland et al., 2005b).) None of these approaches are considered to be preferable compared to ~~provide any advantage over~~ the proposed Stage 1 testing and may even have disadvantages regarding adequacy of mutagen prediction. However a comprehensive review of the performance of Stage 1 genotoxicity assays for prediction of rodent carcinogenicity (excluding a review of the performance of the *in vitro* micronucleus assay) reported positive results in one or more of the three *in vitro* tests for a substantial number of rodent non-carcinogens (as assessed by the Carcinogenicity Potency Database (CPD), National Toxicology Program (NTP), and the International Agency for Research on Cancer (IARC)). Thus the specificity (i.e. correct prediction of negative results for rodent carcinogenicity) was considered to be reasonable for the Ames test (73.9%) but poor for the mammalian cell assays (below 45%).(Kirkland et al., 2005a)

~~31.~~36. The upper limit for sensitivity for prediction of rodent carcinogenicity of for a combination of Ames, mouse lymphoma assay and either micronucleus or chromosome aberration assays was 96%. ~~the recommended three test strategy outlined for Stage 1 was reported to be 90.7%~~ (Kirkland et al., 2005a). The upper limit for sensitivity of a combination of Ames and micronucleus tests was 94.4%. The majority of those rodent carcinogens not detected were considered to induce tumours via a non genotoxic mode of action. A high sensitivity has been a priority of previous genotoxicity testing strategies recommended by the COM(DOH., 2000). The reported specificity (correct identification of non carcinogens) for the combinations of Ames, micronucleus and mouse lymphoma was very low at 5%. The specificity of a combination of Ames and micronucleus tests was 12%. ~~was very low (ca 5%)~~ (Kirkland et al., 2005a). This analysis may be influenced by misleading ~~false~~ positive results in mammalian cell assays in particular the mouse lymphoma assay and the very limited dataset for the *in vitro* micronucleus assay.

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37. 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*. (NB reference Brambilla Toxicology, 196, 1-19, 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). , use of exogenous metabolic activation systems derived from human sources,or recombinant human cytochrome P450 systems as an external activation system. (reference Ku et al Mut Res, 627, 59-77, 2007

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~~32.~~38. There are a number of non-DNA interaction ~~, *in vitro* specific or threshold DNA~~ MOAs by which a chemical may demonstrate ~~an *in vitro*~~ a genotoxic effect that is either not relevant for humans or has a threshold. In both cases a No Observed Effect Concentration (NOEC) can be determined. The COM has reviewed the evidence for a number of threshold MOAs and a general guidance statement is available (www) Threshold MOAs can be g~~G~~enerally ~~these can~~ be considered as 'overload of normal cellular physiology' Investigations of MOA need to be designed on a case by case basis and can be complex to interpret(Kirkland et al., 2007a).

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~~33.~~39. There has thus been considerable debate regarding the highest concentration that should be routinely used in mammalian cell assays. The International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human (ICH) has proposed that the maximum concentration tested for pharmaceuticals should be 1 mM in mammalian cell genotoxicity assays which would have the effect of reducing the number of misleading ~~false~~ positive results due to excessive cytotoxicity. It is also important to note that excessive cytotoxicity may also result in misleading ~~false~~ negative results when pronounced cell cycle delay occurs. A similar conclusion was reached at an international symposium on regulatory aspects of genotoxicity testing(Blakely et al., 2008). However this would not be consistent with the OECD recommendation for a top concentration of 10

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mM in mammalian cell genotoxicity assays (OECD., 1997). The IWGT has reported the preliminary results of an evaluation of published data to investigate concentrations of chemicals which give misleading ~~false~~ positive results in mammalian cell genotoxicity assays. The data suggest a lower top concentration than 10 mM may be acceptable for testing, and although ~~but~~ no definite conclusion on the routine application of a maximum concentration below 10 mM has~~can~~ been reached at present (Parry et al., 2009), a proposal for a new top limit for mammalian cells tests of 1mM or 500 µg/ml (whichever is higher) has been proposed as sufficient to detect all genotoxic carcinogens that are negative in the Ames tests(Kirkland and Fowler, 2010).

~~34.~~40. 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 ~~false~~ positive results (Blakely et al., 2008, Fellows et al., 2008b, Pfuhler, 2009). Many cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems and these cell lines also have ~~,~~ impaired p53 function and altered DNA repair capacity (Kirkland et al., 2007d). There is some evidence to suggest that human lymphocytes are less susceptible to misleading ~~false~~ positives than current used rodent cell lines (e.g. CHO and CHL) and that other cell systems such as the human cell lines HepG2, TK6 and MCL-5 cells and the reconstructed human ~~3D~~ skin models show promise for future use (Kirkland et al., 2007d, Fowler et al., 2010b)~~(Kirkland et al., 2007d)~~. ~~It is also possible to modify test organisms to incorporate metabolic capability (e.g CYP2E1 in Salmonella YG7108pin3ERb$_5$) for specific investigations when considered appropriate (Emmert et al., 2006).~~ The potential impact of method used to assess cytotoxicity may affect the selection of highest concentration tested and potentially the results reached using mammalian cell genotoxicity assays(Kirkland et al., 2007d). It is important that the adequacy of positive results in mammalian cell genotoxicity assays are assessed on a case-by-case basis. Further discussion of these aspects of test assessment is presented below under specific tests.

~~35.~~41. ~~In line with good scientific practice, the results of each *in vitro* assay should be confirmed in an independent experiment. However~~The Committee agree that ~~,~~it is not necessary to undertake independent confirmatory *in vitro* tests provided the following criteria are satisfied:~~for mammalian cell assays this may not be necessary if the following rigorous criteria are met~~ ~~:~~

- there is no doubt as to the quality of the conduct of the test,
- the spacing and range of test substance concentrations leave no chance of missing a positive response~~,~~ ,
- sufficient treatment and sampling times have been used
- the result is not judged to be equivocal by statistical and biological criteria.

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42. While it is accepted that there is no absolute requirement to repeat an *in vitro* assay which has demonstrated a clearly positive result, there is a need to undertake ~~further~~ further genotoxicity testing ~~in an independent assay~~ when an equivocal result is obtained. Such additional genotoxicity tests need to be planned on a case-by-case basis and need not necessarily be undertaken in an ~~Where *in vitro* genotoxicity tests are repeated in a further independent experiment it is not necessary to carry out the second study in an identical fashion~~ identical fashion to the initial experiment. 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 more useful data.

~~6~~43. The use of historical negative control data to aid in the interpretation of genotoxicity test results has been considered particularly in relation to equivocal and small magnitude genotoxic effects (Kirkland et al., 2007b). More recent consideration was undertaken at the 5th IWGT meeting held in Basel during August 17-19, 2009 on the value of historical control data for study acceptance and interpretation of results (Dearfield et al., 2009). Advice was also provided on approaches to establishing historical control data.

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Ideally data should be reported in terms of means and confidence intervals for baseline genotoxicity rather than ranges where outliers can have a disproportionate effect. The data set should be managed so that more recent data carry more weight than older data. Historical negative control data should be 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.

44. All mutagenicity studies should *as far as possible* be carried out to internationally accepted protocols. The Committee recommends dthat the core *in vitro* genotoxicity tests outlined below and in figure 1 should be undertaken. A number of other genotoxicity tests are outlined below which are not part of the core *in vitro* tests but which can provide useful data on a case-by-case basis. The Committee does not recommend the routine use of other *in vitro* assays in which have not been cited in this guidance Stage 1 such as assays for sister chromatid exchange or tests using fungi. The Committee recommends that for new substances all appropriate tests to cover investigation of gene mutation, clastogenicity and aneugenicity and evaluation in Stage 1 should be completed before undertaking any Stage 2 genotoxicity assay.

Discussion of Stage 1 strategy.: Specific core tests

In vitro bacterial tests for gene mutations

45. 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., 1990). The sensitivity for prediction of rodent carcinogenicity based on two independent substantive evaluations is 58.8 and 498.4% respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was reported to be 73.9% and 80.3% and 73.9% (including equivocal results) respectively (Kirkland et al., 2005a, Matthews et al., 2006). 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

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strains being TA 1535, TA1537 (or TA97 or TA97a), TA98, TA100. These strains of *Salmonella typhimurium* may not detect some oxidising mutagens and cross linking agents and thus *Escherichia coli* WP2 (pKM101), WP2*uvrA* or *Salmonella* TA102 should also be used. Testing should be carried out both in the presence and absence of an appropriate exogenous metabolic activation system. However both the repair proficient and repair deficient strains of E coli should be used in those cases where the bacterial assay is the only mutagenicity test being carried out on a given substance, to ensure that cross linking agents are detected.

46. 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)). These methods have not been developed to a point where they can be routinely be used for ~~are at an early stage of development and should not currently be routinely used for~~ regulatory submissions.

In vitro mammalian cell micronucleus assay (IVMN) for clastogenicity and aneuploidy

47~~3~~. The COM recommended in 2000 that equivalent information on clastogenicity and aneuploidy could be obtained from the *in vitro* micronucleus assay compared to classical chromosomal aberration testing in mammalian cells (metaphase analysis). One published comparative analysis of the *in vitro* mincronucleus assay compared to metaphase analysis or the mouse lymphoma assay concluded that the *in vitro* micronucleus assay was as least as adequate as these other two *in vitro* mammalian cell assays (Lorge et al., 2006). The Committee 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 extensive and authorative investigations of the utility of the *in vitro* micronucleus assay so that it is possible to recommend this genotoxicity assay as the first choice test for clastogenicity and aneuploidy detection. Many current published *in vitro* genotoxicity testing strategies recommend that the micronucleus assay and

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metaphase analysis can be considered as equivalent (Cimino, 2006, Eastmond et al., 2009). The sensitivity for prediction of rodent carcinogenicity based on two independent substantive evaluations is 78.7% and 87.3% respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was low and was reported to be 30.8% and 23.1% respectively (Kirkland et al., 2005a, Matthews et al., 2006). It is noted that the data sets used for the assessment of performance of the *in vitro* micronucleus assay were comparatively small compared to other tests considered in this guidance (Kirkland et al., 2005a). An ECVAM (European Centre for the Validation of Alternative Methods) retrospective validation study concluded that the *in vitro* MNT is reliable and can be used as an alternative to the *in vitro* chromosomal aberration assay.(Corvi et al., 2008)

48~~40.~~ The *in vitro* micronucleus assay can be combined with centromere or kinetochore stains, with pancentromeric or chromosome specific centromereic probes using fluorescence in situ hybridization (FISH) as a sensitive way to discriminate between chromosome breaks, chromosome loss and chromosome non-disjunction and polyploidy ~~in combination with the identification of *in vitro* divided cells with the cytokinesis block methodology (CBMN) and of centromeres with pancentrometric or chromosome specific centromeric probes fluorescence *in situ* hybridisation (FISH) is a sensitive easy to score assay which allows assessment of cell proliferation, the discrimination between chromosome breaks, chromosome loss and chromosome non-disjunction and polyploidy~~(Kirsch-Volders et al., 2002). However, binucleate cells obtained with the cytokinesis block methodology (CBMN) will usually be needed for determinations of non-disjunction of chromosomes between daughter nuclei. The CBMN method also allows clear identification that treated and control cells have divided *in vitro* and assessment of cell proliferation. There have been major international collaborative investigations to develop the protocol (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), to investigate the most appropriate methods for measuring cytotoxicity (Fellows et al., 2008a, Lorge et al., 2008, Kirkland,

2010)~~(Fellows et al., 2008a, Lorge et al., 2008)~~ 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). 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), 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 requiring considerable technical skill and is not suitable for routine testing of chemicals for genotoxicity.

495~~1.~~ There~~re is consensus agreement that the~~ use of cytochalasin B to block cell division and generate binucleate cells 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). Scoring of both mononucleated and binucleated cells can be useful for the detection of aneugens (Lorge et al., 2006, Wakata et al., 2006). The binucleate MN assay is more suited to the assessment of genotoxic mechanisms (Parry, 2006). 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 CHI~~L5178Y~~ cells~~)~~ can give more misleading ~~false~~ positive results than~~compared to~~ cell lines proficient for p53 activity ~~(~~such as TK6, HepG2 and human lymphocytes~~)~~ (Fowler, 2009, Fowler et al., 2010a)~~(Fowler, 2009)~~.

5046~~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., 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)~~(Fellows et al., 2008a, Lorge et al., 2008)~~. It has been suggested that ~~Thus~~ using relative cell counts (RCC)

may underestimate cytotoxicity and lead to potentially misleading ~~false~~ positive results (Fowler et al., 2010b). 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 t~~T~~esting beyond 50% survival is not necessary to identify potential mutagens (Fellows et al., 2008a, Lorge et al., 2008). However consensus recommendations embedded in draft OECD guideline 487 indicate that the target range for cytotoxicity in the *in vitro* micronucleus test is 59-50%. Careful selection of toxicity measure has been shown to reduce the potential for misleading ~~false~~ positive results ~~(Fowler, 2009)~~(Fowler et al., 2010b).

51~~473~~. The flow-cytometry-based micronucleus assay (FCMMN) has the potential for increasing reproducibility and increasing turn around time for the micronucleus test (Laingam et al., 2008). However the potential still exists for misleading ~~false~~ positive results from cell processing or from chemical induced apoptosis and necrosis (Laingam et al., 2008). Approaches to overcoming potential misleading ~~false~~ positive results have included use of differential staining of micronuclei (MN) and necrotic and apoptotic cells,(Bryce et al., 2007, Bryce et al., 2008a) use of electronic gating procedures~~, use of p53 mutated cell lines to reduce apoptosis,~~ and use of concurrent assessment of cytotoxicity (Laingam et al., 2008). The FCMMN assay has also be adapted to cell lines which attach to solid surfaces (Bryce et al., 2010).The Committee considered that further development of the FCMMN assay was required before it could 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)

In vitro chromosomal aberration assay in mammalian cells (metaphase analysis) for clastogenicity and aneuploidy

52~~484~~. The *in vitro* chromosome aberration assay in mammalian cells has been widely used in genotoxicity testing for many decades, although only limited

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information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (Aardema et al., 1998). It is noted that polyploidy may not be a reliable indicator for aneugenicity and may result from a number of different genetic changes(MitchelldeG et al., 1995, Galloway, 2000). However the *in vitro* chromosomal aberration assay may provide information on exchanges which are associated with adverse health outcomes. Thus it is important to include the use of chromosome specific centromeric probes fluorescence in situ hybridisation (FISH) to assess the potential for aneuploidy. A wide range of FISH technologies exist for analysis of clastogenic and aneugenic chromosome changes (Maierhofer et al., 2002). The sensitivity for prediction of rodent carcinogenicity based on two independent substantive evaluations is 65.6% and 55.3% ~~and 66.6% (including equivocal results)~~ respectively (Kirkland et al., 2005a, Matthews et al., 2006)~~(Kirkland et al., 2005a, Matthews et al., 2006)~~. Specificity in these two evaluations was reported to be 44.9% and 63.3% respectively (Kirkland et al., 2005a, Matthews et al., 2006)~~(Kirkland et al., 2005a, Matthews et al., 2006)~~. One published evaluation of *in vitro* genotoxicity testing strategies reported that there was no scientific basis to include both a chromosomal aberration and micronucleus assay in addition to Ames and mouse lymphoma assays (Kirkland et al., 2005b). The available data indicate that *in vitro* metaphase analysis -and the *in vitro* micronucleus assay have very similar overall performance as part of a strategy for genotoxicity testing. ~~but metaphase analysis, particularly for the detection of aneuploidy, was technically complex to undertake and thus~~ On balance it is considered ~~it would be~~ preferable to use *in vitro* micronucleus tests for the assessment of clastogenic and aneugenic potential.

In vitro mouse lymphoma assay for gene mutation ~~s, clastogenicity and aneuploidy~~

53~~49~~~~5~~. The Committee 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. ~~a third *in vitro* genotoxicity test should be undertaken in Stage 1. Thus a third assay, comprising an additional gene mutation assay in mammalian cells, should be used, except for compounds for which there is little or no human exposure.~~ Certain mammalian cell gene mutation protocols

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Comment [j3]: Need additional discussion of cytotoxicity evaluation.

Comment [j4]: Is this assay reliable for detection of aneugens?

Comment [j5]: This may change following the June 2010 COM meeting.

that have been widely employed, particularly some of those involving the use of Chinese hamster cells, are considered to be insufficiently sensitive, predominantly on statistical grounds (UKEMS., 1989).~~ The Committee therefore recommends the use of the mouse lymphoma assay as the third test.~~ The sensitivity for prediction of rodent carcinogenicity based on two independent substantive evaluations is 73.1% and 59.3% respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was low and was reported to be 39.0% and 44.2% respectively (Kirkland et al., 2005a, Matthews et al., 2006). These data suggest that the mouse lymphoma assay is not a particularly reliable predictor of rodent carcinogenicity. The retrospective evaluation of studies using more recently developed evaluation criteria (i.e. Global Evaluation Factor (GEF)) may improve the specificity of this assay for correctly identifying rodent non-carcinogens (Matthews et al., 2006).

54~~40~~~~46~~. Since 2000, there has~~ve~~ been considerable development of suitable protocols, negative solvent control data, criteria to define an acceptable positive control response and the use of the GEF and statistical analysis of test results (Clements, 2000, Moore et al., 2003, Kirkland et al., 2007c, Moore et al., 2007). ~~If appropriately used, the~~Some authors have reported that the -mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity, information on recombination, deletion and aneuploidy (Ogawa et al., 2009, Wang et al., 2009)~~(Wang et al., 2009)~~. The committee considers that the detection of aneuploidy with this assay has not been fully substantiated. The mouse lymphoma assay may be used as a follow-up test when a positive in vitro bacterial mutagenicity test has been obtained in situations where animal genotoxicity tests are not permitted(Pfuhler et al., 2010).

Discussion Stage 1: Non-core tests

In vitro assays using human reconstructed skin

55~~1~~~~47~~. ~~There is also a need to develop an enhance *in vitro* genotoxicity testing strategy for chemicals such as cosmetics where no *in vivo* animal tests can be~~

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~~undertaken.~~ 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., 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 comets in reconstructed skin have also been published (Pfuhler et al., 2010). 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 has recently been published.~~None of these assays have been sufficiently well validated for routine screening use in a genotoxicity testing strategy. However the reconstructed skin micronucleus (RSMN) assay using EpiDerm™ shows considerable promise and could be used for investigative purposes on a case-by-case basis.~~

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In vitro alkaline comet assay for DNA damage

56~~248~~. The *in vitro* alkaline comet assay for DNA damage has been proposed as an alternative to undertaking clastogenicity assessment in mammalian cells on the basis that the comet assay results in fewer misleading positive results than chromosomal aberrations due to cytotoxicity(Hartmann et al., 2001, Witt et al., 2007)~~(Witt et al., 2007)~~. It has also been reported that the *in vitro* comet assay can also identify potential mammalian cell gene mutagens (Kirkland and Speit, 2008). Proposals have also been published regarding the use of the comet assay to detect DNA cross-linking agents(Spanswick et al., 2010). There is ~~There is~~ evidence from screening newly synthesised drug candidates that the *in vitro* alkaline comet assay can be used for routine screening of DNA damage and is not confounded by cytotoxicity or compound precipitation (Hartmann et al., 2001). One advantage of the *in vitro* alkaline comet assay is that cell proliferation is not needed and thus any cell type can be used. The comet-FISH assay has been recently developed to provide information on site specific DNA strand breaks (Glei et al., 2009). However, since the comet assay does not detect aneuploidy, and an assessment of

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aneugenicity is an integral part of this genotoxicity testing strategy and hence the *in vitro* comet assay although a useful method to study *in vitro* potential for DNA strand breaks and alkali labile sites is not included in the core tests in Stage 1.

Summary Stage 1

53749. The Committee recommendations for Stage 1 testing incorporates a number of changes compared to are basically similar to those in the 2000 guidelines, the main change(s) being the replacement of the *in vitro* metaphase analysis in mammalian cells with the *in vitro* micronucleus assay (and reduction from three *in vitro* tests to two tests for Stage 1NB for committee discussion on 17 June 2010). Tests should be undertaken in a considered manner according to the best international guidance available to avoid misleading false positive results and data interpreted using appropriate statistical testing and use of historical negative control data. The Committee confirms the need to provide information on gene mutation, clastogenicity and aneugenicity in order to understand genotoxic mode(s) of action (MOA) and to derive conclusions regarding the biological significance of results. Data on MOA are also important in elucidating misleading positive or equivocal results and also with regarding to devising a the strategy for *in vivo* genotoxicity testing. There is a particular need to understand MOA for chemicals which cannot be subject 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. For most test substances the recommended core ree genotoxicity tests in Stage 1are the recommended (*in vitro* bacterial gene mutation test and , *in vitro* micronucleus test and mouse lymphoma assays). In those cases where little or no human exposure is predicted (e.g. chemical intermediates, or some low production volume chemicals) only the first two tests may be appropriate. Such decisions need to be taken on a case-by-case basis by the appropriate regulatory agency. The three rrecommended assays provide , if negative, will provide sufficient information for the genotoxicity assessment of most chemicals. In most instances misleading negative results are due to inadequate exogenous

Comment [j6]: This aspect, namely consideration of two core in vitro tests rather than three tests is subject to further evaluation of utility of MLA.

metabolic activation. However where high, or moderate and prolonged, levels of exposure are expected (e.g. most human medicines) an *in-vivo* assay is recommended to provide additional reassurance. ~~When only two *in vitro* genotoxicity tests are considered necessary it is recommended that these consist of a bacterial assay for gene mutation and an *in vitro* micronucleus assay for clastogenicity and aneugenicity.~~

Stage 2*: In-vivo* genotoxicity tests

Introduction: Overview of initial and supplementary strategies

584~~0~~. Stage 2~~The second stage~~ of the testing strategy involves an assessment of genotoxic activity *in vivo* in somatic tissues and if required germ cells (for the assessment of heritable effects and/or information on hazard classification of mutagens) ~~germ cells (if required)~~ (see Figure 2). The *in vivo* genotoxicity testing strategy outlined below is subdivided into an initial and supplementary stages. The initial *in vivo* genotoxicity testing strategy has to be designed on a case-by case basis and can be used to answer one or more questions relating to; 1) Screening for *in vivo* mutagenic potential. 2) Investigate genotoxicity in tumour target tissue(s), 3) Investigate potential for germ cell genotoxicity, 4) Investigation of mutagenic end point(s) identified in stage 1. It is thus possible for there to be one or more ~~testing~~ separate initial strategies designed to assess questions 1)-4) for a particular substance. This rationale differs from that 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 ~~(peripheral blood in mice)~~ was the preferred first test in almost all cases except for direct acting DNA reactive mutagens where a site of contact test was preferred. The Committee considers that the *in vivo* genotoxicity testing strategy needs to be developed on a case-by-case basis. There was a preference in the 2000 COM guidance for the rat liver UDS assay as a second tissue screening test, which was selected primarily to provide reassurance of absence of *in vivo* genotoxicity when negative results were obtained in an *in vivo* bone marrow MN or CA assay. The selection of rat liver UDS was based largely on experience in use and the availability of an OECD guideline.(DOH., 2000) A revised *in vivo* Stage 2 strategy based on selection of tests to answer one or

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more specific questions on species and/or tissue genotoxicity combined with investigation of particular genotoxic end points and modes of action 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.

5951. Other factors that should be considered when determining an initial *in vivo* genotoxicity testing strategy include whether the testing strategy can be integrated corporated into other regulatory toxicity tests (such as subacute or subchronic toxicity studies). Consideration needs to be given to the nature of the chemical, the results obtained from initial *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). In the animal studies undertaken the routes of exposure should be appropriate to ensure that the substance reaches the target tissue. Thus routes unlikely to give rise to significant absorption in the test animal should be avoided. Confirmatory toxicokinetic studies to measure blood or tissue exposure as appropriate of bone marrow should be undertaken to accompany all when an oral dosing genotoxicity studies bone marrow test has been undertaken in order to assess the adequacy of any negative results obtained.

Comment [j7]: Need to incorporate IWGT Rothfuss paper.

6056. The design of *in vivo* genotoxicity tests should incorporate all appropriate approaches to reduce the number of animals used in such tests. Options for reduction in animal usage include use of one sex only (if supported by metabolism data), restricted administration and sampling times for micronucleus, chromosomal aberration and comet assays, and integration of micronucleus tests into repeat-dose toxicity studies(Pfuhler et al., 2009). It may also be possible to omit positive control administrations in micronucleus and chromosomal aberration tests where the test facility has appropriate historical positive control data (Pfuhler et al., 2009). It may alosalso be possible to combine micronucleus and comet assays into a signlesingle acute administration test (Pfuhler et al., 2009, Vasquez, 2010).

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61572. The approach outlined to Stage 2 in figure 2 takes account of evidence to suggest that *in vivo* comet and transgenic rodent assays have improved

sensitivity and specificity for the identification of rodent carcinogens compared to the rat liver UDS test (Kirkland and Speit, 2008). ~~These endpoints can be integrated together into a single acute study, thus saving animals (ref Pfhulker 2009~~The Committee considers ~~agrees~~ that in addition to screening for mutagenic hazard, a primary focus of *in vivo* genotoxicity testing should include further investigation of the possible ~~confirmatory~~ mode of action as a genotoxic agent~~analysis~~. Thus the initial *in vivo* genotoxicity testing strategy should involve selection of one or more core tests in rodents using Transgenic gene mutation tests, micronucleus tests (accompanied by specific assays for aneuploidy if necessary) or comet DNA damage assays in rodents. In some instances there may be a need to investigate multiple end points before reaching conclusions on *in vivo* mutagenic potential. ~~It may also be possible to undertake the initial *in vivo* testing strategy within routine regulatory toxicity studies.~~

62~~583.~~ Stage 2 *in vivo* genotoxicity tests are needed for ~~tests~~ substances that are positive in any of the *in vitro* Stage 1 genotoxicity tests so as to ascertain whether genotoxic ~~mutagenic~~ activity can be expressed *in vivo*. There are numerous 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 chemicals which have given positive results in one or more *in-vitro* genotoxicity tests. However conclusions on mutagenic hazard and MOA may have to be derived from *in vitro* genotoxicity for substances where no *in vivo* genotoxicity testing is permitted.

63~~594~~. In addition, an *in-vivo* genotoxicity test may detect chemicals that only act *in vivo*, although experience has shown that such compounds are rare (Tweats et al., 2007b). In some instances positive results might be obtained from *in vitro* genotoxicity tests that are adapted to the specific characteristics of the test substance, for example by use of appropriate exogenous metabolising fractions (Muller et al., 2003b). The Committee recommends that for chemicals where exposure is expected to be high, or moderate and sustained, (e.g. most human

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medicines) data from at least one *in vivo* genotoxicity test should be undertaken but only when Stage 1 genotoxicity tests have been completed and assessed.

64055. Positive results in any stage 2 genotoxicity test should be considered for evidence of mode of action (thus do these results confirm data obtained in Stage 1?) and the experiment(s) assessed for evidence of irrelevant positive responses. Examples of irrelevant modes of action in micronucleus tests include compound induced hypothermia in rodents and compound induced increases in cell division of bone marrow erythroblasts (Tweats et al., 2007a, Blakely et al., 2008). If a conclusion is reached that a genotoxic mode(s) of action occurs then the chemical should be considered as an *in vivo* mutagen. MOA data will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The -COM has recently published guidance on possible threshold modes of genotoxicity which can include ; i) involvement of non-DNA targets, (e.g. aneugen inhibition of microtubules) ii) the contribution to protective mechanisms (e.g. repair of DNA adducts formed from small molecular weight alkylating agents and iii) overload of detoxication pathways (e.g. paracetamol) (ref www…)

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Introduction: Germ cell testing

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651. One aspect of the approach to testing outlined in figure 2 is that initial 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 which can also be used if there is a need to evaluate germ cell genotoxicity. There are also a number of specific germ cell genotoxicity assays that might be valuable on a case-by-case basis to provide information on potential for heritable mutagenic effects but these would form part of the supplementary *in vivo* genotoxicity testing strategy.

662. The COM reaffirms that a chemical considered a positive *in vivo* somatic cell mutagen should also be considered as a probable germ cell mutagen unless data can be provided to the contrary. It is noted that there are somerare examples where the mouse bone mouse micronucleus assay does not predict germ cell genotoxicity (Witt et al., 2003, Attia et al., 2005) and examples of

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germ cell mutagens where specific stages of gameotgenosis in males and differences between male and female germ cell genotoxicity have been recorded.(Bishop, 2003).

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Introduction: Supplementary in vivo genotoxicity testing strategy

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67356. A supplementary *in vivo* testing strategy should be undertaken if the results of the initial *in vivo* strategy provide equivocal results or if there is a specific need to investigate heritable effects. This may involve repeating all or aspects of the initial testing strategy, or supplementary investigations (e.g. mode of action investigations, such as DNA adducts or more specific germ cell testing) to investigate aspects of the mutagenicity of the chemical 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, metabolism on the chemical should be considered. ~~Positive results in any part of a supplementary *in vivo* genotoxicity testing strategy should be assessed for evidence of a genotoxic mode of action.~~

68457. If negative results are obtained in the initial *in vivo* testing strategy (supported by appropriate toxicokinetic data on tissue/blood exposure to test substance and metabolites), further supplementary genotoxicity testing would only be needed if a clear positive results had been obtained in a stage 1 *in vitro* genotoxicity tests and there are aspects of the initial strategy (e.g. MOA or need to fully assess germ cell genotoxicity) which have not been fully resolved.

Discussion of Stage 2 initial strategy

General aspects

69558. There are many recent publications debating *in vivo* genotoxicity testing strategies, for example, the German Speaking section of the European Environmental Mutagen Society recommended a single study using combined analysis for micronuclei and ~~with~~ comet induction ~~assay~~ in selected tissues (Pfuhler et al., 2007) and the WHO/IPCS recommended cytogenetics (bone marrow) or gene mutation or alternative test as defined by MOA, chemical class and reactivity (with consideration of factors such as bioavailability and metabolism) (Eastmond et al., 2009). The *in vivo* genotoxicity testing strategy

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recommended by the Committee acknowledges there can be a variety of reasons for undertaking *in vivo* genotoxicity tests and it is important to clearly identify the critical questions (as set out in paragraph above) which have to be answered and develop a strategy accordingly rather than specify a preferred first and second tests. There are comparatively fewer data on the predictivity of *in vivo* genotoxicity assays for rodent carcinogenicity compared to data on the performance of *in vitro* genotoxicity tests and in particular for combinations of *in vivo* genotoxicity assays. Transgenic rodent assays (TGR) and the *in vivo* micronucleus assay exhibited significant complimentarity, consistent with the assessment of different mutagenic end points (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 MOA analysis of *in vitro* mutagenic activity is considerable importance in helping to develop an initial *in vivo* genotoxicity testing strategy. The Committee recommends that the initial *in vivo* genotoxicity testing strategy should be based on one or possibly two tests selected from a relatively limited number of *in vivo* assays that have been specifically designed to provide the optimum amount of information on *in vivo* mutagenic potential of the test substance.

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59. One aspect of the approach to testing outlined in figure 2 is that initial hazard characterisation of germ cell genotoxicity can be included in the initial *in vivo* genotoxicity testing strategy since there are multi tissue *in vivo* genotoxicity assays which can be used if necessary to evaluate germ cells at the same time as part of the initial *in vivo* genotoxicity testing strategy to provide information on other tissues and mode of action. There are a number of specific germ cell genotoxicity assays that might be valuable on a case-by-case basis when specific aspects of germ cell genotoxicity need to be evaluated but these would form part of the supplementary *in vivo* genotoxicity testing strategy.

Discussion of Stage 2 initial strategy.: Specific core tests

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Transgenic rodent mutation assay

70660. There hasve 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 reviewed comprehensively (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. TGR assays can be used to assess gene mutations in all rodent tissues (including germ cells) using all routes of administration (Lambert et al., 2005). 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 available data suggest 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 for detection of weak *in vivo* mutagens. The sensitivity and specificity for prediction of rodent carcinogenicity was reported in the largest evaluation of published literature to be 78% and 69% respectively. The TGR assay would be valuable for all aspects required in the initial *in vivo* genotoxicity testing strategy and particularly to confirm gene mutation as a mode of action. TGR assays have been reported to produced data that are generally compatible with the mouse specific locus test for germ line mutagens (Singer et al., 2006a).

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Rodent bone marrow MN and CA assays

71671. 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 rodent bone marrow micronucleus assay (BMMN) in mice. The bone marrow is readily accessible to chemicals that are present in the blood and a wide range of structurally diverse clastogens has been detected using these methods. The BMMN

micronucleus assay indirectly detects clastogenicity by measuring micronuclei in newly formed cells in the bone marrow (or peripheral blood). It may be used to identify the induction of both structural and numerical aberrations. Micronuclei containing whole chromosomes (as opposed to fragments) can be identified by use of kinetochore or centromeric staining techniques. It should be noted that aneuploidy produced only by chromosome loss can be measured in the bone marrow micronucleus assay. ~~Although most data are available from bone marrow assays, ~~T~~t~~he use of peripheral blood is an alternative approach for both mice (CSGMT, 1995) and in rats (when the youngest fraction of reticulocytes are sampled) (Wakata et al., 1998, Torous et al., 2000, Suzuki et al., 2005a)~~when mice are used. This is not a practical approach in the rat since the spleen removes micronucleated erythrocytes in this species.~~. Clastogenicity may be measured by metaphase analysis in bone marrow of rodents as an alternative approach to the use of the micronucleus assay. The rodent micronucleus assay can be used in the initial *in vivo* genotoxicity strategy for generic screening for *in vivo* mutagenic potential and for assessment of clastogenicity and aneuploidy.

72~~68~~2. There have been developments to incorporate rodent micronucleus assay into routine 28 day subacute toxicity studies which have demonstrated the feasibility of such an approach (Kirshna et al., 1998, Hamada et al., 2001, Madrigal-Bujaidar et al., 2008) and development of a simultaneous liver and peripheral blood micronucleus assay in young rats (Suzuki et al., 2005b)~~(Suzuki et al., 2005)~~. 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 represented average damage during the 30 day period prior to sampling (Witt et al., 2000). The reported sensitivity and specificity for identification of rodent carcinogens based on the EPA GeneTox database for all acute dosing micronucleus tests (all rodent species) is 43.% and 74.9% respectively.

Rodent comet assay

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73693. There have been significant developments with regard to the conduct of *in vivo* alkaline comet assays (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). and Tthere is now consensus agreement on a protocol for most tissues which would be consistent with an OECD guideline (Burlinson et al., 2007). The comet assay can be used in a wide range of species with any tissue including germ cells and can be applied to site-of-contact tests. The comet assay produced positive results for nearly 90% of rodent carcinogens not detected by the rodent bone marrow MN assay (Kirkland and Speit, 2008). The overall specificity, based on a small number of non-carcinogens was 78% (Kirkland and Speit, 2008). The alkaline comet assay identifies double strand breaks and apurinic sites. It measures DNA damage rather than any specific genotoxic mode of action. With regard to the assessment of germ cell genotoxicity measuring DNA effects by the comet assay in sperm requires additional steps for chromatin decondensation. A protocol for standardisation of the germ cell comet assay has not yet been achieved (Speit et al., 2009). The *in vivo* comet assay can be used for all aspects of the initial *in vivo* genotoxicity testing strategy with the exception of mode of action and it is possible to include the comet assay within standard regulatory toxicity tests or within other *in vivo* genotoxicity tests.

Rat liver UDS assay

74064. 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 assay (Kirkland et al., 2005b). An analysis of the prediction of rodent carcinogens not identified by the micronucleus tests indicated that the comet assay was considerably better that the rat liver UDS assay at identifying rodent carcinogens (Kirkland and Speit, 2008). Overall, although the rodent liver UDS and comet assays have a broadly similar profile for detection of DNA damage in the liver (http://www.iacom.org.uk/statements/UDS.htm) -the Committee's preference

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is to use the comet assay rather than rodent liver UDS as a measure of DNA damage.

Discussion of Stage 2 supplementary strategy.

75165. The supplementary *in vivo* testing strategy needs to be considered on a case-by-case basis taking into account all relevant information. It is considered that for most chemicals, a supplementary strategy should be unnecessary but on a case-by case basis specific aspects of MOA (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. Reference is also made 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 Committee is aware that there is the possibility that gender differences in germ cell mutagenesis and genetic risk may exist (Eichenlaub-Ritte et al., 2007). A brief outline of these methods is given in Table 1.

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Table 1*:* *In vivo* assays for consideration in the supplementary *in vivo* strategy.

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA adducts				
^{32}P-postlabelling	DNA adducts	IWGT	Can be applied to all tissues provided sufficient 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;)(MW. et al., 2009;)
Supplementary investigations of germ cell				

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genotoxicity				
Ananalysis for sperm or oocyte clastogenicity/ aneuploidy	Chromosomal /numerical changes		Can provide information on nature of effects and stage(s) of gametogenesis affected	Can provide useful information on MOA (Russo, 2000)
Dominant lethal assay	Chromosomal /gene mutations	OECD	Provides information on heritable genetic changes	Little used. needs relatively large numbers of animals (Adler et al., 1994)
Mouse specific locus test	Gene mutations	EPA	Provides information on heritable genetic changes including information for estimation of mutation frequency	Very rarely used. Needs large numbers of animals
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on heritable genetic changes	Very rarely used. Needs large numbers of animals

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Summary Stage 2 *in vivo* genotoxicity tests.

76265. Stage 2 *in vivo* genotoxicity tests can be undertaken as part of an initial or in a supplementary strategy. The initial *in vivo* genotoxicity testing strategy has to be designed on a case-by case basis and can be used to answer one or more questions relating to; 1) Screening for *in vivo* mutagenic potential. 2) Investigate genotoxicity in tumour target tissue(s), 3) Investigate potential for germ cell genotoxicity, 4) Investigation of mutagenic end point(s) identified in Sstage 1. The first *in vivo* genotoxicity test(s) used in the initial testing strategy could involve Transgenic mutation assay, micronucleus assay or comet assay in rodents. 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) 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 initial strategy further test(s) would only be needed if the chemical was clearly positive in a Stage 1 *in-vitro* genotoxicity test and there were also are aspects of the initial strategy that have not been fully resolved and in the case where an investigation of heritable effects was required. If equivocal results are obtained from the initial *in vivo* strategy, then a supplementary testing strategy is needed. The supplementary additional *in vivo* genotoxicity testing strategy should be devised on a on a case-by case basis. This may involve repeating aspects of the initial *in vivo* genotoxicity testing strategy, or supplementary

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investigations (e.g. mode of action, such as DNA adducts or more detailed consideration of heritable effects ~~germ cell genotoxicity~~). 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 metabolism of the substance, should be considered.

Possible future Developments

77~~3~~~~66~~. The Committee was aware that new assays and toxicogenomic approaches were under development which might be of value within genotoxicity testing. These include gene mutation at the endogenous phopshatidylinositol glycan complementation group A gene (*Pig-A*) as a reporter~~rd~~ gene for mutation in peripheral red blood cells of mammals (Bryce et al., 2008b, Miura et al., 2009) and investigation of instability in expanded simple tandem repeats in male gametes and offspring to evaluate~~ion~~ ~~in~~heritable mutations (Singer et al., 2006b). There have also been rapid developments within the field of toxicogenomics as a method for identifying genotoxic mechanisms. The COM have reviewed data several times during 2008 and 2009 up to the drafting of this guidance statement but currently the evidence does not support the routine use of toxicogenomic approaches to genotoxicity testing http://www.iacom.org.uk/papers/index.htm.

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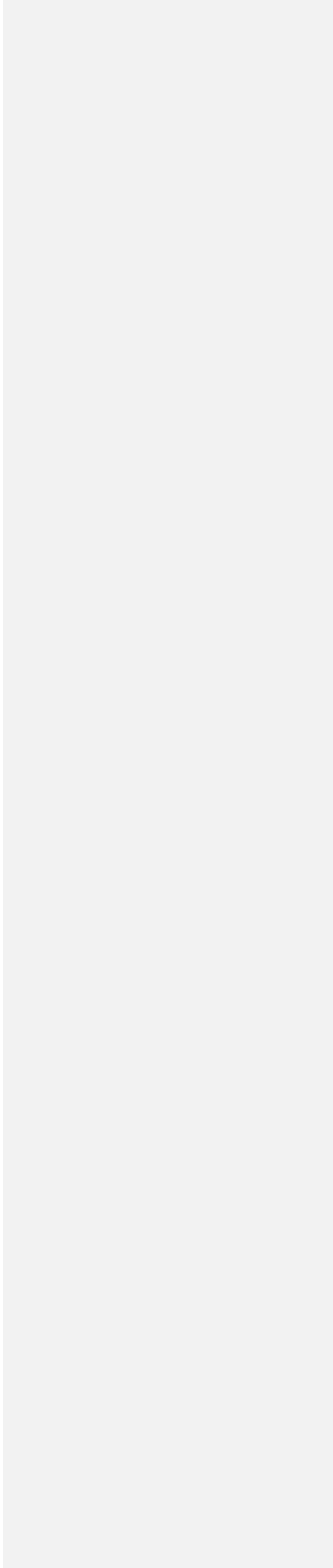

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GLOSSARY

[It is proposed to use the current COT glossary which defines most of the terms cited in the draft COM guidance and to build internet links where appropriate. A number of other terms (DNA stand breaks, polypoidy, recombination) could be taken from ICH guidance. A further number of terms could be taken from the 1989 COM guidance glossary (prokaryotic, eukaryotic) and a further small number of terms would require definitions to be derived,(*in silico*, pre-screening assay, historical negative control, sensitivity, specificity).

Additional terms

DNA damage, due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. While this constitutes only 0.000165% of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as tumour suppressor genes) can impede a cell's ability to carry out its function and appreciably increase the likelihood of tumour formation. The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified. Chemicals that induce DNA damage are referred to as genotoxicants.

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.

Erythrocyte: red blood cell; corpuscle; one of the formed elements 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;, polychromatophilic erythrocyte** one that, on staining, shows shades of blue combined with tinges of pink indicative of an immature erythrocyte.

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.

Historical negative control data*; 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.

The **kinetochore** is the protein structure 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

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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.

*In silico**: Analyses of data sets undertaken by computation models.

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

Pre-screening assay*: High-Throughput procedures designed to provide rapid information on toxicological end points for a large number of compounds.

Sensitivity*: The correct prediction of rodent carcinogens using genotoxicity (mutagenicity) assays based on a defined set of carcinogenicity data (e.g Gold Carcinogenicity Potency database)

Specificity*: 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).

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. Depending on the type of cell division, 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* The value attributed to a particular study (e.g. toxicological or genotoxicity test) within the hazard assessment process for a particular substance. This includes an assessment of the adequacy of the study as well as the reported results

- *= secretariat derived. Others derived from online encyclopedia.

Terms identified from ICH guidance

DNA Strand Break Assay (Comet assay): Alkaline treatment converts certain types of DNA lesions ingot strand breaks that can be detected by the alkaline elution technique 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.

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.

Recombination: Breakage and balanced or unbalance rejoining of DNA

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Terms from the 1989 COM guidance

Prokaryotes: The simplest living organisms namely viruses, bacteria and some blue green algae, in which the genetic material 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 (chromonemal) replication takes place and division and multiplication follow.

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 except the blue-green algae and all animals. Cell division

Terms derived from IPCS draft guidance 2007

(suggest COT definition of chromosomal aberration is expanded to include deletion, duplication, and rearrangement of genetic material)

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.

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

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.

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.

Terms derived from COC guidance 2004

Adduct: A chemical grouping that is covalently bound (see covalent binding) to a large molecule such as DNA (qv) or protein.

Ames test: *In vitro* (qv) assay for bacterial gene mutations (qv) using strains of *Salmonella typhimurium* developed by Ames and his colleagues

DNA (Deoxyribonucleic Acid): The carrier of genetic information for all living organisms except the group of RNA viruses. Each of the 46 chromosomes in normal human cells consists of 2 strands of DNA containing up to 100,000 nucleotides, specific sequences of which make up genes (qv). DNA itself is composed of two interwound chains of linked nucleotides (qv).

Metabolism: Chemical modification of a compound by enzymes within the body, for example by reactions such as hydroxylation (see cytochrome P450), epoxidation or conjugation. Metabolism may result in activation, inactivation, accumulation or excretion of the compound.

Mode of Action: The mode of action of a chemical carcinogen refers to the underlying events involved in the process whereby the chemical induces cancer. 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 action should be distinguished from the term mechanism of action. The latter relates to having sufficient understanding of the molecular basis of the chemical carcinogenesis process to establish causality. Thus mechanism of action is at the other end of a continuum from little or no evidence of mode of action to scientific proof of mechanism of action.

(can adapt this to read MOA for mutation)

Nucleotide: the "building block" of nucleic acids, such as the DNA molecule. A nucleotide consists of one of four bases - adenine, guanine, cytosine, or thymine - attached to a phosphate-sugar group. In DNA the sugar group is deoxyribose, while in RNA (a DNA related molecule which helps to translate genetic information into proteins), the

sugar group is ribose, and the base uracil substitutes for thymine. Each group of three nucleotides in a gene is known as a codon. A nucleic acid is a long chain of nucleotides joined together, and therefore is sometimes referred to as a "polynucleotide."

Threshold: Dose or exposure concentration below which an effect is not expected.

Transgenic animal models: Animals which have extra (exogenous) fragments of DNA incorporated into their genomes. This may include reporter genes to assess *in-vivo* effects such as mutagenicity in transgenic mice containing a recoverable bacterial gene (lacZ or *lac I*). Other transgenic animals may have alterations of specific genes believed to be involved in disease processes (eg cancer). For example strains of mice have been bred which carry an inactivated copy of the p53 tumour suppressor gene (qv) -, or an activated form of the *ras* oncogene which may enhance their susceptibility of the mice to certain types of carcinogenic chemicals.

Note some terms in COT glossary for cancer terms (e.g. tumour) are more limited than in the COC glossary,

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