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Annex 2 to MUT/2010/01

GUIDANCE ON A STRATEGY FOR TESTING OF CHEMICALS FOR GENOTOXICITY

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 Environment, Food and Rural Affairs (defra), the Chemicals Regulatory Directorate (CRD) of the Health and Safety Executive (HSE) (responsible for approval of pesticides and biocides), Veterinary Medicines Directorate (VMD: a defra 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).

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3. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to an agency that does have such a role (e.g. HSE CRD for occupational aspects and for pesticides etc). Its remit is to advise on 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 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 of the various agencies, and it is not intended for the COM guidance to be applied retrospectively.

5. The Committee believes that the approach outlined here will remain valid for several years and will 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 Working 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 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 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. 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 interact with 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 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

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available tests on a particular substance and provides advice on mutagenic hazard.

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. 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.
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

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several different assays. A range of assays has been developed which employs a wide variety of organisms, including bacteria, yeasts and other eukaryotic micro-organisms, 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.

III. General principles of testing strategy

11. The Committee recommends a two-stage genotoxicity testing strategy for the detection of mutagenic hazard. 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 included in regulatory testing strategies (e.g. cosmetics). 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 (Thybauld et al., 2007) and thus a strategy for stage 2 has to be designed based on the nature of mutagenic effects identified in Stage 1.

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12. There 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 stage 2 is more complex and needs to be developed on a case-by-case basis.
13. 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 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. Few chemicals are active only *in vivo* and in such cases this may be due 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).
15. Under the strategy recommended by the Committee, the use of animals in mutagenicity testing is primarily required when it is necessary to investigate whether 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. 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.

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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.

IV Pre-screening considerations prior to genotoxicity testing

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

Physico-chemical and toxicological properties

18. 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 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 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* testing requires estimation of the maximum tolerated dose and consideration of tissue-specific effects.

Structure Activity Relationships

19. 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 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,

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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 approach is DEREK (Deductive Estimation of Risk from Existing Knowledge).(Jacobson-Kram and Contrera, 2007) In contrast (Q)SAR softwares are statistically based programs that 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>

20. Such systems can be useful when a large number of compounds require assessment and prioritisation for biological testing in bacteria. 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 specificity of *Salmonella* bacterial mutagenicity prediction using the FDA MDL QSAR model was 81%

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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).

Pre-screening tests

21. 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 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. Prescreening high through put bacterial tests have been developed using primary DNA damage (*umu* assay), mutations in histidine requirement (fluctuation test), and 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(Westerlink et al., 2009). One research group has proposed a combination of two commercial pre-screening assays (VititoxTM for bacterial mutagenicity and RadarScreen yeast screen for clastogenicity) for rapid screening of compounds and de-selection for genotoxicity (Westerlink et al., 2009).
22. A number of pre-screening genotoxicity tests using mammalian cells have been proposed including oxidative reactions of adducted

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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). None of these pre-screening assays have reached the stage of development where they could routinely be used. The Green Screen has been developed as a pre-screening tool with better concordance with carcinogenicity outcome than regulatory genotoxicity tests (Custer and Sweder, 2008, Knight et al., 2009).

23. Pre-screening 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).

Stage 1: Initial *in vitro* screening

Introduction: Overview of strategy

24. 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 false negatives. 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

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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.

25. As outlined above, Stage 1 involves screening tests for mutagenic activity using *in vitro* methods and comprises a three 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 tests is sufficient to define the chemical as an *in vitro* mutagen. 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 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. There are some occasions where additional *in vitro* genotoxicity testing may be undertaken for chemicals giving a negative response in three

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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 *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 consideration of the appropriate exogenous metabolic activation system (including alternative sources of S-9, other metabolic systems including genetically engineered cell lines)(Ku et al., 2007b).

27. 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 for these chemicals can also give valuable information.
28. 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. 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.

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Discussion of Stage 1 Tests

General aspects

30. The sensitivity of genotoxicity assays have improved over the years and 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 strategy fails to detect mutagenic activity will be due to the absence of appropriate metabolic activity *in vitro*. There have been published proposals which either advocate a 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 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. The sensitivity for prediction of rodent carcinogenicity of the recommended three test strategy outlined for Stage 1 was reported to be 90.7% (Kirkland et al., 2005a). 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) was very low (ca 5%). This analysis may be

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influenced by misleading false positive results in mammalian cell assays and the very limited dataset for the *in vitro* micronucleus assay.

32. There are a number of non-DNA, *in vitro* specific or threshold DNA MOAs by which a chemical may demonstrate an *in vitro* genotoxic effect that is either not relevant for humans or has a No Observed Effect Concentration (NOEC). Generally 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).
33. 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 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 concentration than 10 mM may be acceptable for testing but no definite conclusion on the routine application of a maximum concentration below 10 mM can be reached at present (Parry et al., 2009).
34. 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,

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Fellows et al., 2008b, Pfuhler, 2009). Many cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems, 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 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 3D skin models show promise for future use (Kirkland et al., 2007d). 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. In line with good scientific practice, the results of each *in vitro* assay should be confirmed in an independent experiment. However, 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,
 - the result is not judged to be equivocal by statistical and biological criteria.
35. 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 testing in an independent assay when an equivocal result is obtained. 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 to the initial experiment. Indeed it may be preferable to alter certain aspects of the

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study (e.g. concentration levels investigated) so as to obtain more useful data.

36. 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. 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.
36. All mutagenicity studies should *as far as possible* be carried out to internationally accepted protocols. The Committee does not recommend the routine use of other *in vitro* assays in Stage 1 such as assays for sister chromatid exchange or tests using fungi. The Committee recommends that all appropriate tests 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

37. 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 48.4% and 60.3% (including

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equivocal results) respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was reported to be 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 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), WP2uvrA 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.

38. 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 IITM test (Fluckigetr-Isler et al., 2004)). These methods 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

39. 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* micronucleus 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

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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 authoritative 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 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 87.3% and 80.9% (including equivocal results) respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was reported to be 23.1% and 53.8% (including equivocal results) 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).

40. The *in vitro* micronucleus assay in combination with the identification of *in vitro* divided cells with the cytokinesis block methodology (CBMN) and of centromeres with pancentromeric 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). 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) and initial studies to evaluate a flow cytometric approach to the

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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.

41. There 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 L5178Y cells) can give more misleading false-positive results compared to cell lines proficient for p53 activity (such as TK6, HepG2 and human lymphocytes) (Fowler, 2009).
42. 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). Thus using relative cell counts (RCC) may underestimate

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cytotoxicity and lead to potentially misleading false positive results. 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. Testing beyond 50% survival is not necessary to identify potential mutagens (Fellows et al., 2008a, Lorge et al., 2008). Careful selection of toxicity measure has been shown to reduce the potential for misleading false positive results (Fowler, 2009).

43. The flow-cytometry-based micronucleus assay (CMMN) has the potential for increasing reproducibility and increasing turn around time for the micronucleus test (Laingam et al., 2008). However the potential 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 Committee considered that further development of the FCMMN assay was required before it could be used for regulatory submissions.

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

44. The *in vitro* chromosome aberration assay in mammalian cells has been widely used in genotoxicity testing for many decades, although only limited information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (Aardema et al., 1998). 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

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sensitivity for prediction of rodent carcinogenicity based on two independent substantive evaluations is 55.3% and 66.6% (including equivocal results) respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was reported to be 63.3% and 55.3% (including equivocal results) respectively (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 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 mutations, clastogenicity and aneuploidy

45. The Committee reaffirms the view stated in the 1989 and 2000 guidance, that 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 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 59.3% and 80.8% (including equivocal results) respectively (Kirkland et al., 2005a, Matthews et al., 2006). Specificity in these two evaluations was reported to be 44.2% and 47.6% (including equivocal results) respectively (Kirkland et al., 2005a,

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Matthews et al., 2006). 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).

46. Since 2000, there has been considerable development of suitable protocols, negative solvent control data, criteria to define an acceptable positive control response and the use of the 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 mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity, information on recombination, deletion and aneuploidy (Wang et al., 2009).

Discussion of Stage 1 strategy.: Specific Additional tests

In vitro assays using human reconstructed skin

47. There is also a need to develop an enhanced *in vitro* genotoxicity testing strategy for chemicals such as cosmetics where no *in vivo* animal tests can be undertaken. A number of research groups have developed genotoxicity assays 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). 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.

In vitro alkaline comet assay for DNA damage

48. The *in vitro* alkaline comet assay has been proposed as an alternative to undertaking clastogenicity assessment in mammalian cells (Witt et al., 2007). 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

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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 assessment of 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

49. The Committee recommendations for Stage 1 testing are basically similar to those in the 2000 guidelines, the main change being the replacement of the *in vitro* metaphase analysis in mammalian cells with the *in vitro* micronucleus assay. 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 and to derive conclusions regarding the biological significance of results. Data on MOA are also important regarding 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 instance some useful additional information on genotoxicity may be provided by undertaking *in vitro* tests using reconstructed human skin. For most test substances three genotoxicity tests are recommended (*in vitro* bacterial gene mutation test, *in vitro* micronucleus 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 recommended assays, if negative, will

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provide sufficient information for the assessment of most chemicals. 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

50. The second stage of the testing strategy involves an assessment of genotoxic activity *in vivo* in somatic tissues and germ cells (if required) (see Figure 2). The *in vivo* genotoxicity testing strategy outlined below is subdivided into 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. 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 more

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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.

51. Other factors that should be considered when determining an initial *in vivo* genotoxicity testing strategy include whether the testing strategy can be incorporated 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 exposure of bone marrow should be undertaken when an oral dosing bone marrow test has been undertaken in order to assess the adequacy of any negative results obtained.
52. 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). The Committee agrees that in addition to screening for mutagenic hazard, a primary focus of *in vivo* genotoxicity testing should include confirmatory mode of action analysis. Thus the initial *in vivo* genotoxicity testing strategy should involve selection of one or more 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

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undertake the initial *in vivo* testing strategy within routine regulatory toxicity studies.

53. 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 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.
54. 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 (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 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.
55. 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

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will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The COM reaffirms that a chemical considered a positive somatic mutagen should also be considered as a probable germ cell mutagen. It is noted that there are rare examples where the mouse bone marrow micronucleus assay does not predict germ cell genotoxicity (Witt et al., 2003, Attia et al., 2005).

56. A supplementary *in vivo* testing strategy should be undertaken if the results of the initial *in vivo* strategy provide equivocal results. 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.
57. If negative results are obtained in the initial *in vivo* testing strategy, further supplementary genotoxicity testing would only be needed if a clear positive result 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

58. 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 with comet assay in selected tissues (Pfuhler et al., 2007) and the WHO/IPCS recommended cytogenetics (bone marrow) or gene mutation or

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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 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 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 and in particular for combinations of *in vivo* genotoxicity assays. Transgenic rodent assays (TGR) and the *in vivo* micronucleus assay exhibited significant complimentary, 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.

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

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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 tests

Transgenic rodent mutation assay

60. There has been a significant increase in the number of studies undertaken with transgenic rodent mutation (TGR) assays published since the COM guidance published in 2000. These have been reviewed comprehensively (Lambert et al., 2005). There are sufficient data to assess the performance of the MutaTM 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

61. 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). 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, the use of peripheral blood is an alternative approach 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.
62. 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., 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

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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 43.1% and 74.9% respectively.

Rodent comet assay

63. 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) and there 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

64. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver (Kennelly et al., 1993). The

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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 than the rat liver UDS assay at identifying rodent carcinogens (Kirkland and Speit, 2008). Overall the Committee's preference is to use the comet assay rather than rodent liver UDS as a measure of DNA damage.

Discussion of Stage 2 supplementary strategy.

65. 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 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.

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

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA adducts				
³²P-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	Interpretation of results can be complex. Involves handling high-activity ³² P. (Phillips et al., 2000)

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			enrichment technique used.	
Covalent binding to DNA A variety of methods can be used such as those involving radioactive decay 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). (MW. et al., 2009;)
Supplementary investigations of germ cell genotoxicity				
An analysis 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

Summary Stage 2 *in vivo* genotoxicity tests.

65. 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 stage 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, consider the evidence for genotoxic mode of action and

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check 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 are aspects of the initial strategy that have not been fully resolved. If equivocal results are obtained, then a supplementary testing strategy is needed. The 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 investigations (e.g. mode of action, such as DNA adducts or more detailed consideration of 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, and available information on toxicokinetics metabolism of the substance, should be considered.

Possible future Developments

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 phosphatidylinositol glycan complementation group A gene (*Pig-A*) as a reported 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 evaluation inheritable 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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References

- Aardema M, Albertini S, Arni P, Henderson L, Kirsch-Volders M, Mackay J, Sarrif D, Stringer D, Taalman R (Aneuploidy: a report of an ECETOC task force. . Mutation Research 410:3-79.1998).
- Aardema M, Snyder R, Spicer C, Divi K, Morita T, Mauthe R, Gibson D, Soelter S, Curry P, Thybaud V, Lorenzon G, Lorge E (SFTG international collaborative study on in vitro micronucleus test. III. Using CHO cells. Mutation Research 607:61-87.2006).
- Ackerman J, Sharma R, Hitchcock J, Hayashi T, Nagai Y, Li S, Lu S, Miret J, Tang K, Spence F, Aubrecht J (Inter-laboratory evaluation of the bioluminescent Salmonella reverse mutation assay using 10 model chemicals. Mutagenesis 24:433-438.2009.).
- Adler I, Shelby M, Bootman J, Favor J, Generoso W, Pacchierotti F, Shibuya T, Tanaka N (Summary report of the Working Group on Mammalian Germ Cell Tests. Mutation Research 312:313-318.1994).
- Ashby E, Paton D (The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 different human carcinogen exposures. . Mutation Research 286:3-74.1993).
- Attia S, Badary O, Hamada S, de Angelis M, Adler I (Othovanadate increased the frequency of aneuploid mouse sperm without micronucleus induction in mouse bone marrow erythrocytes at the same dose level. Mutation Research 583:158-167.2005).
- Aubrecht J, Oowski J, Persaud P, Cheung J, Ackerman J, Lopes S, Ku W (Bioluminescent Salmonella reverse mutation assay: a screen for detecting mutagenicity with high throughput attributes. Mutagenesis 22:335-342.2007).
- Benfenati E, Benigni R, Demarini D, Helma C, Kirkland D, Martin T, Mazzatorta P, Ouédraogo-Arras G, Richard A, Schilter B, Schoonen W, Snyder R, Yang C (Predictive models for carcinogenicity and mutagenicity: frameworks, state-of-the-art, and perspectives. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27:57-90.2009.).
- Benigni R, Bossa C (Structure alerts for carcinogenicity, and the Salmonella assay system: a novel insight through the chemical relational databases technology. Mutation Research 659:248-261.2008).
- Benigni R, Netzva T, Benfenati E, Bossa C, Franke R, Helma C, Hulzebos E, Marchant C, Richard A, Woo Y-T, Yang C (The Expanding ROle of Predictive Toxicology: An Update on the (Q)SAR Models for Mutagens and Carcinogens. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 25:53-97.2007).
- Blakely D, Galloway S, Kirkland D, MacGregor J (Regulatory aspects of genotoxicity testing: from hazard identification to risk assessment. Mutation Research 657:84-90.2008).
- Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G (The *in vivo* comet assay: use and status in genotoxicity testing. Mutagenesis 20:245-254.2005).
- Bryce S, Avlasevich S, Bemis J, Lukamowicz M, Elhajouji A, Van Goethem F, De Boeck M, Beerens D, Aerts H, van Gompel J, Collins J, Ellis P, White A, Lynch A, Dertinger S (Interlaboratory evaluation of a flow cytometric, high content *in vitro* micronucleus assay. Mutation Research 650:181-195.2008a).
- Bryce S, Bemis J, Avlasevich S, Dertinger S (In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutation Research 78-91.2007).
- Bryce S, Benis J, Dertinger S (In Vivo Mutation Assay based on the Endogenous *Pig-a* Locus. Environmental and Molecular Mutagenesis 49:256-264.2008b).
- Burlinson B, Tice R, Speit G, Agurell E, Brendler-Schwaab S, Collins A, Escobar P, Honma M, Kumaravel T, Nakajima M, Sasaki Y, Thybaud V, Uno Y, Vasquez M, Hartmann A (Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup. Mutation Research 627:31-35.2007).
- Cariello N, Wilson J, Britt B, Wedd D, Burlinson B, Gombar V (Comparison of the computer programs DEREK and TOPKAT to predict bacterial mutagenicity. Mutagenesis 17:321-329.2002).
- Cimino M (Comparative Overview of Current International Strategies and Guidelines for Genetic Toxicology Testing for Regulatory Purposes. Environmental and Molecular Mutagenesis 47:362-390.2006).

This is a draft paper for discussion. It should not be quoted, cited or reproduced. The text does not represent an agreed view of COM.

- Clare M, Lorenzon G, Akhurst L, Marzin D, van Delft J, Montero R, Botta A, Bertens A, Cinelli S, Thybaud V, Lorge E (SFTG international collaborative study on vitro micronucleus test II. Using human lymphocytes. *Mutation Research* 607:37-60.2006).
- Claxton L, Stewart-Hook V, Warren S (Methods for the Spiral *Salmonella* mutagenicity assay including specilaised applications. . *Mutation Research* 488:241-257.2001).
- Clements J (The Mouse Lymphoma Assay. *Mutation Research* 455:97-110.2000).
- Combes R, Grindon C, Cronin M, Roberts D, Garrod J (Proposed Integrated Decision-tree Testing Strategies for Mutagenicity and Carcinogenicity in Relation to the EU REACH Legislation. . *ATLA* 35:267-287.2007).
- Contrera J, Mathews E, Kruhlak N, Benz R (In silico screening of Chemicals for Bacterial Mutagenicity Using Electropological E-state indices and MDL QSAR Software. *Regulatory Toxicology Pharmacology* 43:313-323.2005).
- Contrera J, Matthews E, Kruhlak N, Benz R (In Silico Screening of Chemicals for Genetic Toxicity Using MDL-QSA, Nonparametric Discriminant Analysis, E-State, Connectivity and Molecular Property Descriptors. *Toxicology Mechanisms and Methods* 18:207-216.2008).
- Curren R, Mun G, Gibson D, Aardema M (Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm™). *Mutation Research* 607:192-204.2006).
- Custer L, Sweder K (The role of Genetic Toxicology in Drug Discovery and Optimization. *Current Drug Metabolism* 9:978-985.2008).
- Dean S, Brooks T, Burlinson B, Mirsalis J, Myhr B, Recio L, Thybaud V (Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing for *in vivo* for the detection of site-of-contact mutagens. *Mutagenesis* 14:141-151.1999).
- Dearfield K, Hayashi M, Jacobson-Kram D, Kasper P, Lovell D, Martus H, Thybaud V (Topic 5: Historical Control data. 5th International Working Group on Genotoxicity Testing.2009).
- DOH. (Department of Health. Report on Health and Social Subjects, 35. Guidelines for the testing of chemicals for Mutagenicity. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. London, HMSO.1989).
- DOH. (Department of Health. Guidance for the testing of chemicals for Mutagenicity. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment.2000).
- Eastmond D, Hartwig A, Anderson D, Anwar W, Cimino M, Dobrev I, Douglas G, Nohmi T, Phillips D, Vickers C (Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonised Scheme. *Mutagenesis* 24:341-349.2009).
- Eichenlaub-Ritte U, Adler I, Carere A, Pacchierotti F (Gender differences in germ cell mutagenesis and genetic risk. *Environmental Research* 104:22-36.2007).
- Fellows M, O'Donovan M, Lorge E, Kirkland D (Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test II. Practical aspects with toxic agents. *Mutation Research* 655:4-21.2008a).
- Fellows M, O'Donovan M, Lorge E, Kirkland D (Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test II: Practical aspects with toxic agents. *Mutation Research* 655:4-21.2008b).
- Fenech M (Cytokinesis-block micronucleus assay evolves into a 'cytome' assay for chromosomal instability, mitotic dysfunction and cell death. *Mutation Research* 600:58-66.2006).
- Fenech M (Cytokinesis-block micronucleus assay. *Nature Protocols* 2:1084-1104.2007).
- Flamand N, Marrot L, Belaidi J-P, Bourouf L, Dourille E, Feltes M, Meunier J-R (Development of genotoxicity test procedures with Episkin® a reconstructed human skin model: Towards new tools for *in vitro* risk assessment of dermally applied compounds? *Mutation Research* 606:39-51.2006).
- Fluckigetr-Isler S, Baumeister M, Braun K, Gervais V, Hasler-Nguyen N, Reimann R, Van Gompel J, Wunderlich H-G, Engelhardt G (Assessment of the performance of the Ames II™ assay: a collaborative study with 19 coded compounds. *Mutation Research* 558:181-197.2004).
- Fowler P (Industrial Genotoxicity Group, November 2009. Reduction of False/Misleading Positives in *in-vitro* genetic toxicology testing: Importance of cell selection and toxicity measure. .2009).
- Garas A, Webb E, Pillay V, MacPhee D, Denny W, Zeller H, Cotton R (A novel and simple method of screening compounds for interaction with DNA: A validation study. *Mutation Research* 678:20-29.2009).
- Garriott M, Phelps J, Hoffman W (A protocol for the *in vitro* micronucleus test I. Contributions to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanisms of action and different levels of activity. *Mutation Research* 517:123-134.2002).

This is a draft paper for discussion. It should not be quoted, cited or reproduced. The text does not represent an agreed view of COM.

- Gatehouse D, Rowland I, Wilcox P, Calander R, Forster R (Bacterial Mutation Assays in Basic Mutagenicity Tests. UKEMS Recommended Procedures. D Kirkland (ed) UKEMS Report. Cambridge U. Press pp 13-61.1990).
- Gealy R, Wright-Bourque J, Kraynak A, McKelvey T, Barnum J, Storer R (Validation of a high-throughput *in vitro* alkaline elution/rat hepatocyte assay for DNA damage. Mutation Research 629:49-63.2007).
- Glei M, Hovhannisyan G, Pool-Zobel B (Use of Comet-FISH in the study of DNA damage and repair: Review. Mutation Research 681:33-43.2009).
- Grant S, Zhang Y, Klopman G, Rosenkranz H (Modeling the mouse lymphoma forward mutational assay: the Gene-Tox program database. . Mutation Research 465:201-229.2000).
- Hamada S, Sutou S, Morita T, Wakata A, Asanami S, Hosoya S, Ozawa S, Kondo K, Nakajima M, Shimada H, Osawa K, Kondo Y, Asano N, Sato S, Tamura H, Yajima N, Marshall R, Moore C, Blakely D, Schectman L, Weaver J, Torous D, Proudlock R, Ito S, Namiki C, Hayashi M (Evaluation of the Rodent Micronucleus Assay by a 28-Day Treatment Protocol: Summary of the 13th Collaborative Study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)-Mammalian Mutagenicity Study Group (MMS). Environmental and Molecular Mutagenesis 37:93-110.2001).
- Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice R (Recommendations for conducting the *in vivo* alkaline Comet assay. Mutagenesis 18:45-51.2003).
- Hartmann A, Kiskinis E, Fjallman A, Suter W (Influence of cytotoxicity and compound precipitation on test results in the alkaline comet assay. . Mutation Research 497:199-212.2001).
- Helleday T, Johansson F, Jenssen D (The DRAG Test: an Assay for Detection of genotoxic Damage. ATLA 29:233-241.2001).
- Hiramoto K, Nasuhara A, Michikoshi K, Kato T, Kikugawa K (DNA strand-breaking activity and mutagenicity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4-H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. . Mutation Research 395 47-56.1997).
- Hu T, Kaluzhny Y, Mun G, Barnett B, Karetsky V, Wilt N, Klausner M, Curren R, Aardema M (Intralaboratory and interlaboratory evaluation of the EpiDerm™ 3D human reconstructed skin micronucleus assay. Mutation Research 673:100-108.2009).
- Jacobson-Kram D, Contrera J (Genetic Toxicity Assessment: Employing the Best Science for Human Safety part I: Early Screening for Potential Human Mutagens. Toxicological Sciences 96:16-20.2007).
- Kasper P, Uno Y, Mauthe R, Asano N, Douglas G, Matthews E, Moore M, Mueller L, Nakajima M, Singer T, Speit G (Follow-up testing of rodent carcinogens not positive in the standard genotoxicity test battery: IWGT workgroup report. Mutation Research 627:106-116.2007).
- Kennelly J, Water R, Ashby J, Lefeure P, Burlinson B, Benford D, Dean S, Mitchell I d (In vivo rat liver UDS assay. In Supplementary Mutagenicity Tests. UKEMS Report. Cambridge University Press pp 52-77.1993).
- Kirkland D, Aardema M, Banduhn N, Carmichael P, Fautz R, Meunier J, Pfuhrer S (*In vitro* approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive *in vitro* genotoxicity results. Mutagenesis 22:161-175.2007a).
- Kirkland D, Aardema M, Henderson L, Muller L (Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens 1. Sensitivity, specificity and relative predictivity. Mutation Research 584:1-256.2005a).
- Kirkland D, Hayashi M, Jacobson-Kram D, Kasper P, MacGregor J, Muller L, Uno Y (Summary of major conclusions from the 4th IWGT, San Francisco, 9-10 September, 2005. Mutation Research 627:5-9.2007b).
- Kirkland D, Hayashi M, Jacobson-Kram D, Kasper P, MacGregor J, Muller L, Uno Y (Summary of major conclusions from the 4th IWGT, San Francisco, 9-10, 2005. Mutation Research 627:5-9.2007c).
- Kirkland D, Henderson L, Marzin D, Muller L, Parry J, Speit G, Tweats D, Williams G (Testing strategies in mutagenicity and genetic toxicology: An appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutation Research 588:88-105.2005b).
- Kirkland D, Pfuhrer S, Tweats D, Aardema M, Corvi R, Darroudi F, Elhajouji A, Glatt H, Hastwell P, Hayashi M, Kasper P, Kirchner S, Lynch A, Marzin D, Muarici D, Meunier J-R, Muller L, Nohynek G, Parry J, Parry E, Thybaud V, Tice R, van Benthem J, Vanparys P, White P (How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid

This is a draft paper for discussion. It should not be quoted, cited or reproduced. The text does not represent an agreed view of COM.

- unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutation Research* 628:31-55.2007d).
- Kirkland D, Speit G (Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. *Mutation Research* 654:114-132.2008).
- Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate MJ, Kirchner S, Lorge E, Morita T, Norppa H, Surralles J, Vanhauwaert A, Wakata A (Report from the *in vitro* micronucleus assay workshop group. *Mutation Research* 540:153-163.2003).
- Kirsch-Volders M, Vanhauwaert A, DeBoeck M, Decordier I (Importance of detecting numerical versus structural chromosome aberrations. *Mutation Research* 504:137-148.2002).
- Kirshna G, Urda G, Theiss J (Principles and Practices of Integrating Genotoxicity Evaluation Into Routine Toxicology Studies: A Pharmaceutical Industry perspective. *Environmental and Molecular Mutagenesis* 32:115-120.1998).
- Knight A, Little S, Houck K, Dix D, Judson R, Richard A, McCarroll N, Akerman G, Yang C, Birrell L, Walmsley R (Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast™ chemicals. *Regulatory Toxicology Pharmacology* 55:188-199.2009).
- Ku W, Aubrecht J, Mauthe R, Schiestl R, Fornace Jr A (Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part VII: Why Not Start with a Single Test: A Transformational Alternative to Genotoxicity Hazard and Risk Assessment. *Toxicological Sciences* 99:20-25.2007a).
- Ku W, Bigger A, Brambilla G, Glatt H, Gocke E, Guzzie P, Hakura A, Honma M, Martus H, Scott Obach R, Roberts S (Strategy for genotoxicity testing-Metabolic considerations. *Mutation Research* 627:59-77.2007b).
- Laingam S, Frosio S, Humpage A (Flow-cytometric analysis of *in vitro* micronucleus formation: Comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cells. *Mutation Research* 656:19-26.2008).
- Lambert I, Singer T, Boucher S, Douglas G (Detailed review of transgenic rodent mutation assays. *Mutation Research* 590:1-280.2005).
- Lorge E, Hayashi M, Albertini S, Kirkland D (Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. *Mutation Research* 655:1-3.2008).
- Lorge E, Thybaud V, Aardema M, Oliver J, Wakata A, Lorenzon G, Marzin D (SFTG international collaborative study on the *in vitro* micronucleus test I. General conditions and overall conclusions of the study. *Mutation Research* 607:13-36.2006).
- Madrigal-Bujaidar E, Madrigal-Santillan E, Alvarez-Gonzalez I, Baez R, Marquez P (Micronuclei Induced by Imipramine and Desipramine in Mice: A Subchronic Study. *Basic & Clinical Pharmacology & Toxicology* 103:569-573.2008).
- Maierhofer C, Jentsch I, lederer GF, C., Speicher M (Multicolor FISH in two and three dimensions for clastogenic analysis. *Mutagenesis* 17:523-527.2002).
- Matthews E, Kruhlak N, Cimino M, Benz R, Contrera J (An analysis of genetic toxicology, reproductive and developmental toxicity and carcinogenicity data: II Identification of genotoxicants, reprotoxicants, and carcinogens using *in silico* methods. *Regulatory Toxicology Pharmacology* 44:97-110.2006).
- Miller J, Vlasakova K, Glaab W, Skopek T (A low volume, high-throughput forward mutation assay in *Salmonella typhimurium* based on fluorouracil resistance. *Mutation Research* 578:210-224.2005).
- Miura D, Dobrovolsky V, Kimoto T, Kasahara Y, Heflich R (Accumulation and persistence of *Pig-A* mutant peripheral red blood cells following treatment of rats with a single and split doses of *N*-ethyl-*N*-nitrosourea. *Mutation Research* 677:86-92.2009).
- Moore M, Honma M, Clements J, Bolcsfoldi G, Burlinson B, Cifone M, Clarke J, Clay P, Doppalapudi R, Fellows M, Gollapudi B, Hou S, Jenkinson P, Muster W, Pant K, Kidd D, Lorge E, Lloyd M, Myhr B, O'Donovan M, Riach C, Stankowski Jr L, Thakur A, van Goethem F (Mouse Lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. *Mutation Research* 627:36-40.2007).
- Moore M, Honma M, Clements J, Bolcsfoldi G, Cifone M, Delongchamp R, Fellows M, Gollapudi B, Jenkinson P, Kirby P, Kirchner S, Muster W, Myhr B, O'Donovan M, Oliver J, Omori T, Oudelhkim M-C, Pant K, Preston R, Riach C, San R, Stankowski Jr L, Thakur A, Wakuri S, Yoshimura I (Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: International Workshop on Genotoxicity Tests Workgroup Report-Plymouth, UK 2002. *Mutation Research* 540:127-140.2003).

This is a draft paper for discussion. It should not be quoted, cited or reproduced. The text does not represent an agreed view of COM.

- Muller L, Blakely D, Dearfield K, Galloway S, Guzzie P, Hayashi M, Kasper P, Kirkland D, MacGregor J, Parry J, Schectman L, Smith A, Tanaka N, Tweats D, Yamasaki H (Strategy for genotoxicity and stratification of genotoxicity test results- report of initial activities of the IWGT Expert Group. *Mutation Research* 540:177-181.2003a).
- Muller L, Blakely D, Dearfield K, Galloway S, Guzzie P, Hayashi M, Kasper P, Kirkland D, MacGregor J, Parry J, Schectman L, Smith A, Tanaka N, Tweats D, Yamasaki H (Strategy for genotoxicity testing and stratification of genotoxicity test results-report on initial activities of the IWGT Expert Group. *Mutation Research* 540:177-181.2003b).
- Mun G, Aardema M, Hu T, Barnett B, Kaluzhny Y, Klausner M, Karetsky V, Dahl E, Curren R (Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay. *Mutation Research* 673:92-99.2009).
- MW. H, Boogaard P, Cadet J, Farmer P, Kim J, Martin E, Persaud R, Shuker D (Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. . *Critical Reviews in Toxicology* 39:679-694.2009;).
- OECD. (Organisation for Economic Co-operation and Development. Ninth addendum to the OECD guidelines for the testing of chemicals. Update of Section 4, Health Effects. Revised guidelines 424, 471, 473, 474, 475, 476, 483, 486. .1997).
- OECD. (OECD Series on testing and Assessment. Number 49. The report from the Expert Group on (Quantitative) Structure-Activity Relationships [(Q)SARs] on the principles for the validation of (Q)SARs. (2nd Meeting of the Ad hoc Expert group on QSARs, OECD Headquarters, 20-21 September 2004. JT00176183, ENV/JM/MONO(2004)24, 17-Dec-2005.2004).
- Oliver J, Meunier J-R, Awogi T, Elhajouji A, Ouldelhkim M-C, Bichet N, Thybaud V, Lorenzon G, Marzin D, Lorge E (SFTG international collaborative study on in vitro micronucleus test V. Using L5178Y cells. *Mutation Research* 607:125-152.2006).
- Parry J (The use of the *in vitro* micronucleus assay to detect and assess the aneugenic activity of chemicals. *Mutation Research* 607:5-8.2006).
- Parry J, Parry E, Phrakinkham P, Corvi R (5th International Workshop on Genotoxicity testing, August 17-19, 2009. Analysis of published data for top concentration of cytotoxicity testing in mammalian genotoxicity testing. .2009).
- Pfuhler S (5th International Workshop on Genotoxicity testing, August 17-19, 2009. Topic 3: In vitro test approaches with better predictivity. ICEM, Florence, August 20-25, 2009.2009).
- Pfuhler S, Albertini S, Fautz R, Herbold B, Madle S, Utesch D, Poth A (Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part IV: Recommendation of a Working Group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a Simple and Straightforward Approach to Genotoxicity Testing. *Toxicological Sciences* 97:237-240.2007).
- Phelps J, Garriott M, Hoffman W (A protocol for the in vitro micronucleus test II. Contributions to the validation of a protocol suitable for regulatory submissions from an examination of 10 chemicals with different mechanisms of action and different levels of activity. *Mutation Research* 521:103-112.2002).
- Phillips D, Farmer P, Beland F, Nath R, Poirier M, Reddy M, Turteltaub K (Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ Mol Mutagen* 35:222-233.2000).
- Reifferscheid G, Arndt C, Schmid C (Further Developmen of the β -lactamase Mutagen Assay and Evaluation by Comparison with Ames Fluctuation Tests and the umu Test. *Environmental and Molecular Mutagenesis* 46:126-139.2005).
- Roithfuss A, Steger-Hartmann T, Heinrich N, Wichard J (Conspirational Prediction of the Chromosome-Damaging Potential of Chemicals. *Chem Res Toxicol* 19:1313-1319.2006).
- Russo A (In vivo cytogenetics in mammalian germ cells. *Mutation Research* 455:167-189.2000).
- Singer T, Lambert I, Williams A, Douglas G, Yaulk C (Detection of induced male germline mutation: Correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutation Research* 598:164-193.2006a).
- Singer T, Lambert I, Williams A, Douglas G, Yaulk C (Detection of induced male germline mutation: Correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutation Research* 598:164-193.2006b).

This is a draft paper for discussion. It should not be quoted, cited or reproduced. The text does not represent an agreed view of COM.

- Snyder R, Smith A (Computational prediction of genotoxicity: room for improvement. *Drug Discovery Today (Biosilico)* 10:1119-1124.2005).
- Speit G, Vasquez M, Hartmann A (The comet assay as an indicator test for germ cell genotoxicity. *Mutation Research* 681:3-12. .2009).
- Suzuki H, Ikeda N, Kobayashi K, Terashima Y, Shimada Y, Suzuki T, Hagiwara T, Hatakeyama S, Nagaoka K, Yoshida J, Saito Y, Tanaka J, Hayashi M (Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). . *Mutation Research* 583:133-145.2005).
- Thybaud V, Dean S, Nohmi T, de Boer J, Douglas G, Glickman B, Gorelick N, Heddle J, Heflich R, Lambert I, Martus H, Mirsalis J, Suzuki T, Yajima N (In vivo transgenic mutation assays. *Mutation Research* 540:141-151.2003).
- Thybaud V, Aardema M, Clements J, Dearfield K, Galloway S, Hayashi M, Jacobson-Kram D, Kirkland D, MacGregor J, Marzin D, Ohymama W, Schuler M, Suzuki H, Zeiger E (Strategy for genotoxicity testing: Hazard identification and risk assessment in relation *in vitro* testing. *Mutation Research* 627:41-58.2007).
- Tweats D, Blakely D, Heflich R, Jacobs A, Jacobsen S, Morita T, Nohmi T, O'Donovan M, Sasaki Y, Sofuni T, Tice R (Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards. *Mutation Research* 627:78-91.2007a).
- Tweats D, Blakely D, Heflich R, Jacobs A, Jacobsen S, Morita T, Nohmi T, O'Donovan M, Sasaki Y, Sofuni T, Tice R (Report of the IWGT working group on strategy/interpretation for regulatory *in vivo* tests. II. Identification of *in vivo*-only positive compounds in the bone marrow micronucleus test. *Mutation Research* 627:92-105.2007b).
- UKEMS. (Statistical evaluation of mutagenicity test data. UKEMS sub-committee on guidelines for mutagenicity testing. Report . Part III. Editor Kirkland DJ. Published Cambridge University Press.1989).
- Wakata A, Matsuoka A, Yamakage K, Yoshida J, Kubo K, Kobayashi K, Senjyu N, Itoh S, Miyajima H, Hamada S, Nishida S, Araki H, Yamamura E, Matsui A, Thybaud V, Lorenzon G, Marzin D, Lorge E (SFTG international collaborative study on in vitro micronucleus test IV. Using CHL cells. *Mutation Research* 607:88-124.2006).
- Wang J, Sawyer J, Chen L, Chen T, Honma M, Mei N, Moore M (The Mouse Lymphoma Assay Detects Recombination, Deletion and Aneuploidy. *Toxicological Sciences* 109:96-105.2009).
- Westerlink W, Stevenson J, Lauwers A, Griffioen G, Horbach G, Schoonen W (Evaluation of the Vitotox™ and RadarScreen assays for rapid assessment of genotoxicity in the early research phase of drug development. *Mutation Research* 676:113-130.2009).
- Witt I, Plappert U, de Wall H, Hartmann A (Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation part III. The Comet Assay as an Alternative to *In vitro* Clastogenicity Tests for Early Drug Candidate Selection. *Toxicological Sciences* 97:21-26.2007).
- Witt K, Hughes L, Burka L, McFee A, Mathews J, Black S, Bishop J (Mouse bone marrow micronucleus test results do not predict the germ cell mutagenicity of N-hydroymethylacrylamide in the mouse dominant lethal assay. *Environ Mol Mutagen* 41:111-120.2003).
- Witt K, Knapton A, Wehr C, Hook G, Mirsalis J, Shelby M, MacGregor J (Micronucleated Erythrocyte Frequency in Peripheral Blood of B6C3F₁ Mice from Short-Term, prechronic, and Chronic Studies of the NTP Carcinogenesis Bioassay Program. *Environmental and Molecular Mutagenesis* 36:163-194.2000).
- Worth A, Bassan A, Gallegos A, Netzeva T, Patlewicz G, Pavan M, Tsakovska I, Vracko M (The Characterisation of (Quantitative) Structure-Activity Relationships: Preliminary Guidance. Report from European Commission Joint Research Centre, EUR 21866 EN, 2005.2005).
- Zeiger E, Ashby J, Bakale G, Enslein K, Klopman G, Rosenkranz H. Prediction of Salmonella mutagenicity. *Mutagenesis* 11:471-484.1996).