

**MUT/2020/03**

**COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER  
PRODUCTS AND THE ENVIRONMENT (COM)**

**COM Guidance Series Update**

**Background**

Continued consideration and comments of the Guidance update.

Members are asked to complete review of this latest draft as attached.

**Secretariat  
October 2019**

# GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALS

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## GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALS.

### Executive Summary

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agencies with advice on the most suitable approaches to testing chemical substances for genotoxicity. The COM published guidance in 1981, 1989, 2000 and again in 2011. This document incorporates some significant changes and reports the COM views regarding the most appropriate strategy for genotoxicity testing reached in 2019, bringing the guidance document up to date.
2. It should be noted that in this updated guidance, several key areas have been identified as potentially requiring frequent updating, due to their fast-moving nature. To facilitate such updates, standalone documents have been prepared outlining the currently available status of test strategies for germ cell mutagens (paras X) and the use of Quantitative Structure Activity Relationship (QSAR) modelling (paras x), both of which were included in the previous version of the guidance document. In addition, standalone documents have been prepared detailing the use of 3D tissue models for genotoxicity testing and test guidance strategies for manufactured nanomaterials. Both of these areas were not included in the previous version of the guidance document and are now briefly detailed in **paras XX and XX**.
3. The COM recommends a staged approach to testing:
  - **Stage 0** consists of preliminary considerations which include physico-chemical properties of the test chemical substance, Structure Activity Relationships (SAR), and information from screening tests<sup>1</sup>. However, data from SAR and screening tests should not overrule test data from adequately designed and conducted genotoxicity tests.
  - **Stage 1** consists of *in vitro* genotoxicity tests. The COM recommends a core-test battery of the Ames test combined with the *in vitro* micronucleus test. This combination provides information on three types of genetic damage for which data are required (namely, gene mutation, chromosomal damage and aneuploidy) and gives appropriate sensitivity to detect chemical genotoxins. As also supported by the OECD, the COM consider that there is no need to

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<sup>1</sup> Note that the terms 'test' and 'assay' are used interchangeably throughout the document to reflect naming conventions.

independently replicate adequately designed and conducted core *in vitro* tests which are either clearly negative or clearly positive. The strategy document also considers the contribution that can be made by a number of non-core *in vitro* tests.

- **Stage 2** consists of *in vivo* genotoxicity tests. A case-by-case strategy should be developed to answer one or more of the following specific queries;

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

4. The core tests in Stage 2 are the rodent micronucleus/chromosome aberration assays for aneuploidy and clastogenicity, the transgenic rodent gene mutation assay and the rodent comet assay for DNA damage.

5. Usually, negative results obtained in a carefully selected *in vivo* test (possibly studying more than one endpoint and tissue) will be sufficient to address positive results found *in vitro*. However, a further test(s) may be needed if some of the genotoxic effects seen in Stage 1 *in vitro* tests had not been adequately studied *in vivo* (e.g. the chemical affects multiple mutagenic end-points), or other aspects of the genotoxic potential of the chemical had not been fully resolved (e.g. in the case where an investigation of heritable effects was required). The strategy document also considers the contribution that can be made by a number of non-core *in vivo* tests. In most instances information from core *in vivo* tests is sufficient to evaluate the *in vivo* mutagenicity of chemical substances. A supplementary *in vivo* test strategy can provide additional information on a case-by-case basis, to investigate aspects such as further characterisation of germ cell genotoxicity, and DNA adduct data which can provide information to elucidate the mode of genotoxic action of carcinogenic chemicals.

### I. Preface

6. The COM is an independent expert advisory committee whose members are appointed by the secretary of state for health and social care and the chair of the Food Standards Agency (FSA) following an appointments exercise involving public advertisement. Members serve in their own capacity as independent experts and observe a published code of practice including principles relating to the declaration of possible conflicting interests.

7. The remit of the COM is to advise any UK government departments and agencies with an interest in the safety of chemicals across various sectors on the human health aspects of the mutagenicity and genotoxicity of chemicals (these terms are defined for the purposes of this guidance document in paragraphs 9 - 10 below). The Secretariat is provided by Public Health England (PHE), who lead, and the FSA. Other government departments with an interest provide assessors to the COM; these are specifically from the Department of Health and Social Care (DHSC), the Department of Environment, Food and Rural Affairs (Defra), the Chemicals Regulation Division (CRD) of the Health and Safety Executive (HSE) (responsible for legislation regulating chemicals, pesticides, biocides and detergents), the Environment Agency (EA), the Veterinary Medicines Directorate (VMD; a Defra agency responsible for the licensing of veterinary drugs) and the Medicines and Healthcare products Regulatory Agency (MHRA; a DHSC 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.

8. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to a body that does have such a role (e.g. HSE CRD for occupational aspects and for pesticides etc). Its remit is to advise on the human health aspects of mutagenicity and genotoxicity of chemicals, and this may involve advice on a specific chemical, and also on testing strategies and research. This guidance document focuses on testing strategies for chemicals for which there are no available genotoxicity data. Separate guidance on a strategy for the genotoxicity testing and mutagenic hazard assessment of chemicals with inadequate genotoxicity data was published in 2011 ([https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/315802/strategy\\_for\\_chemicals\\_with\\_inadequate\\_genotoxicity\\_data.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/315802/strategy_for_chemicals_with_inadequate_genotoxicity_data.pdf)). Throughout this guidance the COM has referred to the genotoxicity testing of chemical(s) which refers to a specified chemical or material including any additive necessary to preserve its stability and any impurity deriving from the process used.

## DRAFT DOCUMENT FOR DISCUSSION

The COM usually provides advice on a specific chemical which can be equated to a single chemical or compound (<http://www.hse.gov.uk/reach/definitions.htm#substance>). Nanomaterials are not considered in this guidance. Provision of advice on radiation aspects is not within the scope of the COM.

9. The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with potential mutagenic and genotoxic hazards (inherent properties of chemicals) or risk (the likelihood of mutagenic or genotoxic effects occurring after a given exposure to a chemical) and to present recommendations for genotoxicity testing. In practice the bulk of the work of the COM relates to assessing genotoxicity tests and providing advice on the genotoxic hazard of chemicals.

10. In the context of testing strategies, the COM first published guidelines for the testing of chemicals for mutagenicity in 1981, and these were revised in 1989 (DOH, 1989) and 2000 (DOH, 2000). These provided guidance to the relevant government departments and agencies on best practice for testing at those times. The rationale developed by COM in 2000 (DOH, 2000), particularly in relation to the testing of all potential mutagenic endpoints, was adopted by the International Workshops on Genotoxicity Testing (IWGT) (Muller et al., 2003). The need for guidance to be periodically updated, to reflect advances in development and validation of methods, was recognised and substantially revised guidance was published in 2011 (DOH, 2011). This version of the guidance brings the 2011 document up to date. The strategy outlined in this guidance is considered to be the most scientifically appropriate given available methods and recognises the need to avoid the use of live animals where practical and validated alternative methods are available. Testing strategies, the same or similar to those outlined in the 2011 COM guidance, have been adopted by some regulatory bodies, including the European Food Safety Authority (EFSA) (EFSA, 2011) and included in the notes on Guidance from the Scientific Committee on Consumer Safety (SCCS) (SCCS, 2016) and in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation (ECHA, 2017).

11. The COM believes that the approach outlined presents an overview of the core principles of genotoxicity testing and will remain valid for several years. It is acknowledged that existing national or international testing strategies will be at different stages of review and hence inconsistencies are expected. The COM guidance is not intended to supersede or replace existing national or international sector-specific genotoxicity testing strategies (e.g. those recommended for pharmaceuticals by the

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 2011) for chemicals assessed under REACH Regulations (EC1906/2006) (ECHA, 2017) or by EFSA (EFSA, 2011).

## II. Introduction

12. The COM last published guidance on a strategy for the testing of chemicals for mutagenic potential in 2011 (DOH, 2011). The guidance outlined in 2011 was based on the development of new approaches to identifying genotoxic hazards *in vitro* including new approaches to identify misleading positive results and evaluate target organ genotoxicity *in vivo*. There is also a need for a testing strategy which can encompass chemicals such as cosmetics where no animal tests are permitted under European Union (EU) law. It is the objective of this paper to set out a scientifically valid testing strategy comprising those methods which the COM believe to be the most informative with regards to the detection of genotoxic hazard and (when possible) are well validated. There is no discussion of methods which experience has shown to be suboptimal in determining genotoxicity. Details of methodologies are not given since they are provided in the Organisation for Economic Cooperation and Development (OECD) test guidelines, the EU Test Methods Regulation (EC 440/2008) and the IWGT guidance.

13. The genome can be damaged in a variety of ways either spontaneously or from exposure to genotoxic agents. The term “mutagenic” refers to the ability of a chemical to induce a permanent change in the amount or structure of the genetic material of an organism, which may result in an heritable change in the characteristics of the organism. Chemicals inducing mutations are referred to as mutagens (they are mutagenic). These alterations may involve individual genes, blocks of genes, or whole chromosomes. Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of larger changes, including deletions and rearrangements of DNA. The potential to induce mutation is measured in test systems that detect a broader range of genetic changes than simply mutation – they measure genotoxicity. Mutagenicity is accepted as a key event in carcinogenicity. Epigenetic changes, that could also be heritable, fall outside the scope of this guidance.

14. Genotoxicity refers to interaction with, or damage to, DNA and/or other cellular components which regulate the fidelity of the genome. It is a broad term that, as well as mutation includes damage to DNA such as the production of DNA adducts, by the chemical itself or its metabolites. Cells have the capacity to protect themselves from such potentially lethal or mutagenic genotoxic effects by many repair processes and



therefore many genotoxic events do not become evident as mutations. However, the capacity to damage the genome (genotoxicity) is an indicator of potential mutagenicity. Thus, some methods that measure genotoxicity do not provide direct evidence of heritable mutation.

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

16. The COM reaffirms its view, published in 1989, 2000 and 2011, that there is currently no single validated assay that can provide comprehensive information on all three types of genetic damage and thus it is necessary to subject a given test chemical to several different assays. The range of assays discussed in this document include those using prokaryotes (bacteria) and mammalian cells *in vitro*, and whole mammals, where effects in a wide range of target organs including germ cells can be measured. Assays may be classified on the basis of genetic end-points (e.g. gene mutation, clastogenicity, aneugenicity and tests for DNA damage) or by consideration of the different phylogenetic levels (e.g. bacteria, and mammalian cell) represented and also in mammals by the tissues or target organs studied.

### III Significance of Chemical-Induced Mutation for Human Health

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

18. A separate statement discussing the significance of chemical-induced mutation to human health was published in 2012 (<https://www.gov.uk/government/publications/the-significance-of-chemical-induced-mutation-for-human-health>).

#### IV. General Principles of Testing Strategy

19. The COM recommends a two-stage testing strategy (Stages 1 and 2) for the detection of the genotoxic hazard of chemicals which can be supported by appropriate preliminary screening tests and/or *in silico* data (Stage 0).

20. Initial testing for genotoxic potential in Stage 1 is based upon two core *in vitro* tests that are chosen to provide information on gene mutation, clastogenicity and aneuploidy, with case-by-case additional testing and investigation depending on the results of these initial genotoxicity tests. All *in vitro* tests should be designed to provide the best chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system (S9 - see glossary); (b) the ability of the compound or its metabolite(s) to reach the target DNA and/or targets such as the cell division apparatus, and; (c) the ability of the genetic test system to detect the given type of genotoxic event. Where international guidance is available, the assays should be carried out to conform to those internationally recognised protocols e.g. as published by the OECD, the IWGT and in the EU test methods Regulation (EC 440/2008). The same approach to testing can be used for chemicals where *in vivo* genotoxicity testing is not permitted (e.g. cosmetics). Investigations regarding MoGA are important to derive conclusions on biological relevance of genotoxicity test results, to aid in overall risk assessment, and to inform on the strategy for *in vivo* tests. This is of particular importance for those chemicals where no *in vivo* genotoxicity testing is permitted.

21. For most chemicals, results from the two Stage 1 core tests should be sufficient to reach a conclusion on the presence or absence of mutagenic potential. However, in some instances, even when Stage 1 tests are negative, regulatory authorities may require consideration of the need for *in vivo* Stage 2 testing particularly where exposure is considered to be high, or moderate and prolonged (e.g. most human medicines), or where there is a chemical class precedent (i.e. structural relationship) of positive *in vivo* genotoxicity data. Guidance on the level of exposure which equates to high, moderate or prolonged is beyond the remit of the COM.

22. Stage 2 consists of a number of *in vivo* tests designed to investigate whether *in vitro* genotoxic activity including specific 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 tumours detected in carcinogenicity bioassays) or in germ cells. Few chemicals are active only *in vivo* and in such cases this may be due to a number of factors such as metabolic differences, the influence of gut flora, higher exposures *in*

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

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

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

25. Under the strategy recommended by COM, the use of animals in genotoxicity testing is primarily required when it is necessary to investigate whether genotoxic activity detected in Stage 1 *in vitro* is reproduced *in vivo*, to study target organ genotoxicity (for example involvement of genotoxicity in rodent tumours) and to evaluate the potential for heritable mutagenic effects. Genotoxicity testing using animals, when required by guidance, should be carried out when there is no suitable alternative, and the minimum number of animals should be used, consistent with obtaining valid results. If feasible, studies can be conducted as an adjunct to single or repeat dose toxicity studies. The COM supports current and future developments to replace, refine or reduce the need for animals, consistent with the principles of the 3Rs<sup>2</sup>.

### V Genotoxicity Testing Strategy

26. The COM guidance provides a strategy for testing chemicals where no genotoxicity data are available. Test chemicals may also contain impurities at varying levels which may exhibit genotoxic activity. Separate guidance on the genotoxicity assessment of impurities is available at <https://www.gov.uk/government/publications/genotoxicity-assessment-of-impurities-in-chemical-substances>. The assessment and control of genotoxic impurities is the subject of an ICH Guideline (M7) ([http://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Multidiscipli](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidiscipli)

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<sup>2</sup> <https://www.nc3rs.org.uk/the-3rs>

[nary/M7/M7\\_Step\\_4.pdf](#)) and ICH M7(R1)  
[http://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Multidisciplinary/M7/M7\\_R1\\_Addendum\\_Step\\_4\\_31Mar2017.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M7/M7_R1_Addendum_Step_4_31Mar2017.pdf)

27. The strategy recommended in the following sections is concerned with testing for genotoxic activity of chemicals and does not specifically address complex mixtures of chemicals. Since the publication of the COM guidance in 2000, assessments of the performance of QSAR approaches, screening tests and genotoxicity assays (both individually and in combinations) regarding the prediction of rodent carcinogenicity have been published (Kirkland et al., 2005a; Mathews et al., 2006; ). Reference to these publications can provide an insight into the performance of the *in vitro* genotoxicity assays specifically in relation to the particular data sets analysed and the end points considered, predominantly rodent carcinogenicity but also *in vivo* genotoxicity (Kirkland et al, 2011). Relevant sensitivity and specificity data and assay performance assessments have been summarised in Annex 1, and are discussed further in Annex 3, for information and are cited where appropriate in the text below. Overall, the older available data suggest that mammalian cell assays did not perform well at discriminating between rodent carcinogens and non-carcinogens (e.g. Kirkland et al, 2005a, 2007c; Mathews et al., 2006). However, experience suggests that mammalian cell tests conducted and interpreted according to current recommendations perform more robustly (Clarke et al., 2012).

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

28. The intrinsic chemical and toxicological properties of the test chemical must be considered before devising the genotoxicity testing programme. Manufactured nanomaterials present particular considerations with regards to genotoxicity testing which are discussed in a separate document 'Test Guidance Strategies for Genotoxicity Testing of Manufactured nanomaterials' (COM, 2019).

#### Physico-chemical and Toxicological Properties

29. The physico-chemical properties of the test chemical (for example, acid dissociation constant (pKa), partition coefficient, solubility, volatility and stability in, and potential reactions with, solvents/vehicles) and its purity can affect the ease of conduct and results of *in vitro* tests. For example, the tolerance of cells to acidic chemicals can be enhanced by neutralisation but this may affect the inherent reactivity of chemicals to DNA (Hiramoto et al., 1997). Potential reactions of the test chemical with solvent /vehicle should also be considered (e.g. cisplatin reacts with dimethyl sulfoxide

(DMSO)) (Fischer et al., 2008). Alternatively, low solubility may limit the feasibility of undertaking some or all of the *in vitro* mutagenicity tests recommended in this strategy. The potential for auto-oxidation of the test chemical in the culture medium can also affect the outcome of *in vitro* genotoxicity tests (Long et al., 2007). It is noteworthy that the toxic properties of test chemicals, such as target organ effects, or irritancy/corrosivity in contact with skin or mucous membranes and their toxicokinetics and metabolism will influence the choice of route of administration and the highest dose level achievable in Stage 2 *in vivo* mutagenicity tests.

### Quantitative Structure Activity Relationships (QSAR)

30. Whether the test chemical would be expected to have mutagenic potential may be assessed from its chemical structure, which may provide structural alerts for mutagenicity. A range of QSARs have been developed to predict genotoxicity and these are detailed in full in the COM document 'Use of QSAR models to predict genotoxicity (COM, 2019). The COM has previously agreed that where no genotoxicity data are available, initial assessment of potential genotoxicity can be based on the publicly available QSAR models. COM considered updated information on QSAR models in February 2018, which formed the basis of the document 'Use of QSAR models to predict genotoxicity (COM, 2019). It was concluded that whilst it remained useful to evaluate data generated from QSAR models, in particular as a negative predictor for screening purposes, no changes to the previously recommended guidance were warranted.

31. Overall, QSAR approaches for the prediction of genotoxic activity can be a valuable tool to aid in the high throughput screening of compounds, the provision of assessments for chemicals for which no genotoxicity test data are available and also prioritisation for genotoxicity testing. QSAR can also aid in the interpretation of genetic toxicology tests. Expert judgement is needed when reaching conclusions on mutagenic hazard on the basis of QSAR information alone, and such predictions cannot replace the need to undertake the *in vitro* and *in vivo* genotoxicity tests required to derive conclusions on mutagenic hazard. . In reaching conclusions, data from well conducted *in vitro* or *in vivo* genotoxicity tests should be attributed a much higher weight of evidence than QSAR predictions, although all information should be assessed on a case-by-case basis.

### Screening Tests

32. There are a number of initiatives which have attempted to combine data mining *in silico* approaches with high throughput tests to develop approaches to screening

large numbers of novel chemicals (Benfenati et al., 2009). In this guidance, genotoxicity screening tests refers to high throughput tests which have been designed to be rapid, economical, reproducible, require only small amounts of test chemicals (typically below 50 mg) and have a high concordance with comparator genotoxicity end points in genotoxicity tests; these tests are also often referred to as pre-screening tests. High throughput bacterial tests have been developed using combinations of *Salmonella* tester strains (Ames II™), primary DNA damage (*umu* assay), mutations in ampicillinase gene (MutaGen assay), bioluminescence or 5-fluorouracil resistance (Ackerman et al., 2009.; Aubrecht et al., 2007; Kamber et al., 2009 ; Miller et al., 2005; Reifferscheid et al., 2005). The performance of several of these tests used for screening purposes in the pharmaceutical industry has been reviewed (Escobar et al., 2013). Other screening systems cited in the literature include DNA repair activity in yeast cells (Westerink et al., 2009). One research group has proposed a combination of two commercial screening assays (Vitotox™ for bacterial mutagenicity and RadarScreen yeast screen for clastogenicity) for rapid screening of compounds (Westerink et al., 2009).

33. A number of genotoxicity screening tests using *in vitro* systems have been proposed, including alkaline elution using rat hepatocytes (Gealy et al., 2007), the detection of DNA damage (via p53 or GADD45a activation, GreenScreen) in cell lines (Knight et al., 2009) and differential survival in DNA repair proficient and deficient cell lines (Helleday et al., 2001). A screening test for genotoxicity using HepG2 cells (metabolically competent with wild type p53 genotype) based on four different luciferase-reporter assays has been published. The authors claim, based on a small dataset, a high sensitivity for identification of genotoxicity when used in combination with the commercially available systems (Vitotox™ and RadarScreen) (Westerink et al., 2010). None of these genotoxicity screening tests have reached the stage of development where they could routinely be used to replace data generated from *in vitro* genotoxicity testing. The predominant use of high throughput screening tests is as an aid in prioritisation of compounds for development undertaken by industry. The COM reviewed the GADD45a-GFP assay and it was agreed that currently, it is most suited as part of a battery of high throughput screening (COM minutes March 2010) ([http://webarchive.nationalarchives.gov.uk/20140506144744/http://www.iacom.org.uk/meetings/documents/COMminsMarch2010finalforinternet\\_000.pdf](http://webarchive.nationalarchives.gov.uk/20140506144744/http://www.iacom.org.uk/meetings/documents/COMminsMarch2010finalforinternet_000.pdf)).

34. The ToxTracker assay, a cell line based genotoxicity test, was reviewed by the COM in 2014. The test system comprises 6 reporter cell lines in which genes reflecting key signalling pathways had been cloned into mouse embryonic stem cells (Hendriks

et al., 2012; Hendriks et al., 2011). The different cell lines are capable of detecting chemicals that may cause genotoxic or oxidative damage (e.g. the *Bsc12* cell line is preferentially responsive to genotoxins and *Srxn1* cell line is preferentially responsive to pro-oxidants). Members considered that the assay would be most usefully employed as a biomarker assay or for determining potential mode of action. It should be noted that because 6 different cell lines are treated, Toxtracker cannot really be considered a high-throughput screening assay. However, the potential application in a genotoxicity testing strategy where *in vivo* testing is not permitted (e.g. cosmetics) was acknowledged (COM minutes March 2014) ([https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/690196/COM\\_meeting\\_minutes\\_March\\_2014.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/690196/COM_meeting_minutes_March_2014.pdf)).

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

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

#### Overview of strategy

36. The COM concluded in 1989 and 2000 that it was appropriate to concentrate on a relatively small number of assays, using validated, sensitive methods particularly chosen to **avoid misleading negative results**. Two important parts of the revised Stage 1 strategy include using appropriate tests to gain an insight into the nature of the genotoxic effects of a test chemical and also to **avoid misleading positive results** (when compared to *in vivo* testing results). Misleading positive results have been reported for certain *in vitro* mammalian monolayer cell assays such as the chromosomal aberration test, micronucleus test and mouse lymphoma assay (Kirkland et al., 2005a, 2007c; Fowler et al., 2012a, b; Pfuhler et al., 2011), particularly when multiple test systems were used.

37. Misleading positive results are considered to be caused by a number of factors, including the use of cell lines of rodent origin (e.g. V79, CHO, CL) that partially lack normal cell cycle control, have limited metabolic capacity (even with the addition of S9) and do not mimic site-specific metabolic capacity (Reus et al., 2013). The development of 3D tissue models is hoped to reduce the number of misleading positive findings and improve the accuracy of predictions due to their improved metabolic capacity and proximity to *in vivo* gene expression and protein functions (Andres et al., 2012;



Barcham et al., 2018). The current state of the science for 3D model development and validation is discussed in '3D Tissue Models for Genotoxicity Testing' (COM, 2019).

38. As outlined above in **paragraph 20**, Stage 1 involves tests for genotoxic activity using *in vitro* methods and comprises a two test core system; namely an *in vitro* bacterial test for gene mutation (Ames test) and an *in vitro* micronucleus test (MNvit), with the objective of assessing genotoxic potential by investigating three different end points (gene mutation, structural chromosomal damage and changes in chromosome number). The rationale for this test strategy is given in Annex 3. **A clear positive result in either of these two core tests is sufficient to define the chemical as an *in vitro* genotoxin, although further *in vitro* and/or *in vivo* testing may be undertaken to understand the relevance of the positive results.** The Committee considers that this revised strategy, developed in 2011, allows for efficient identification of all genotoxic-end points but, by reducing the number of mammalian cell tests from that recommended by COM in 2000, and following improved methodologies, the risk of misleading positive results (i.e. when compared with *in vivo* genotoxicity data) is decreased.

39. Additional investigations of chemicals which give positive or repeated equivocal results in Stage 1 tests can include an assessment of mode(s) of *in vitro* genotoxic action. There are a number of reasons (discussed in **paragraphs 46-49**) why positive results in *in vitro* genotoxicity tests might occur by mode(s) of action not relevant to human health hazard assessment. Such MoGA evaluation *in vitro* is particularly relevant for those chemicals (e.g. cosmetics) where there is a regulatory constraint which precludes the use of *in vivo* genotoxicity assays in the testing strategy. The COM does not recommend the use of *in vitro* genotoxicity assays that have not been considered in detail in this guidance or for which OECD guidelines have been deleted. This includes assays for sister chromatid exchange, the *in vitro* Unscheduled DNA Synthesis (UDS) assay, the *in vitro* comet assay or tests using fungi. A table of genotoxic endpoints detected by each assay cited in Stage 1 of this strategy is given in Annex 2.

40. For chemicals which give equivocal results or repeated small positive effects, when considering biological relevance, it is important to consider evidence of reproducibility in the same assay or in different assays detecting similar effects, and the magnitude of the induced genotoxic effect in relation to historical negative control data, and then consider whether further *in vitro* genotoxicity testing is needed (Hayashi et al., 2011; Kirkland et al., 2007a). Further consideration of SAR data for these chemicals may also give valuable information (Dearfield et al., 2010).



41. If clear negative results are obtained in both core *in vitro* tests undertaken, it can generally be concluded that the chemical has no genotoxic activity. However, there are some occasions when additional *in vitro* and/or *in vivo* genotoxicity testing may be undertaken for chemicals giving a negative response in the two *in vitro* core genotoxicity tests. For example, in situations where tumours are found in rodents, where the *in vitro* metabolic activation systems are not optimal or where there are human-specific metabolites, there may be a need for further genotoxicity assessment. A further testing strategy would have to be designed on a case-by-case basis (Kirkland et al., 2007b; Muller et al., 2003). An IWGT working group has published guidance on this topic (Kasper et al., 2007). An important part of any additional *in vitro* strategy should be consideration of the appropriate exogenous metabolic activation system (including alternative sources of S9 or other metabolic systems including genetically engineered cell lines, see paragraph 48) (Ku et al., 2007b). Further information on *in vivo* genotoxicity testing of such test chemicals is provided in Stage 2 of this strategy.

42. Information from other combinations of genotoxicity tests which may include one or more non-core tests outlined below in paragraphs 67-72 may also give adequate data on all three end-points on a case-by-case basis. *In vitro* genotoxicity tests (micronucleus scoring and comet) using human reconstructed skin may provide useful information on *in vitro* mutagenic hazard in circumstances where *in vivo* testing is not permitted, or when extensive dermal exposure is anticipated (e.g. cosmetic ingredients) (Aardema et al., 2013; Chapman et al., 2014; Roy et al., 2016; Reisinger et al., 2018).

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

### Discussion of Stage 1 Tests- General Aspects

44. The conduct of genotoxicity assays has improved over time and the overall sensitivity of *in vitro* testing strategies regarding prediction of rodent carcinogens is very high (Kirkland et al., 2007c; Kirkland et al., 2005a). Proposals have been published for genotoxicity testing advocating a single *in vitro* genotoxicity test (Ku et al., 2007a) or a complex approach involving up to six *in vitro* genotoxicity tests (SCCNFP, 2003). The latter approach has been critically evaluated (Kirkland et al.,

2005b) and COM considers that neither is considered preferable to the proposed Stage 1 core testing. Although the sensitivity (producing positive results with carcinogens) for rodent carcinogenicity of a battery of Stage 1 tests was very high, the specificity (producing negative results with non-carcinogens) was poor (Kirkland et al., 2005a; Kirkland et al., 2007c; Pfuhler et al., 2011). Possible reasons for the poor specificity have been discussed by various working groups e.g., see (Kirkland et al., 2007c). A comprehensive review of the performance of Stage 1 genotoxicity assays for prediction of rodent carcinogenicity reported positive results in one or more *in vitro* tests for a substantial number of rodent non-carcinogens (as assessed by the Carcinogenic Potency Database (CPDB), National Toxicology Program (NTP), and the International Agency for Research on Cancer (IARC). Thus the specificity (i.e. correct identification of rodent non-carcinogens) was considered to be reasonable for the Ames test (74%) but poor for the mammalian cell assays (below 45%) particularly when multiple assays were performed (Elespuru et al., 2009; Kirkland et al., 2005a). Many reasons for low specificity have been proposed, particularly for mammalian cells; for example, the use of high-concentrations, cytotoxicity, prolonged exposure, overloading defence mechanisms, lack of detoxification capacity. The influence of such confounding effects leading to indirect mechanisms of genotoxicity, has been widely recognised (Kirsch-Volders, 2003; Müller, 2000; Pratt, 2003; Kirkland et al., 2007a)

45. A later analysis on the sensitivity of a combination of Ames test and MNvit test to detect rodent carcinogens and *in vivo* genotoxicants is summarised and discussed in Annex 3 Table 1 (Kirkland et al., 2011). It is difficult to draw precise conclusions from the available sensitivity and specificity data since the databases of chemicals used vary. However, these data do show that mammalian cell genotoxicity tests can have low specificity and that combinations of *in vitro* genotoxicity tests result in high sensitivity for rodent carcinogens and *in vivo* genotoxicants. High sensitivity has always been a priority of genotoxicity testing strategies recommended by the COM (DOH, 2000, 2011). COM evaluated the use of *in vitro* genotoxicity tests to predict rodent carcinogens and *in vivo* genotoxicants in June 2010 and concluded **that there is no convincing evidence that any [relevant or DNA reactive] rodent carcinogen or *in vivo* genotoxicant would fail to be detected by using an *in vitro* genotoxicity test battery consisting of Ames and MNvit tests** (<http://webarchive.nationalarchives.gov.uk/20140506144308/http://www.iacom.org.uk/meetings/index.htm>).

46. It is most likely that one of the few occasions where *in vitro* test strategies fail to detect mutagenic activity (i.e. misleading negative results) could be explained by the

absence of appropriate metabolic activity *in vitro* (Brambilla and Martelli, 2004) or that the test chemical does not reach the cells. Approaches to resolving potential inadequacies in metabolic activation include structure based metabolism predictions, use of genetically modified target organisms (e.g. CYP2E1 in *Salmonella* YG7108pin3ERb<sub>5</sub>) (Emmert et al., 2006), the use of exogenous metabolic activation systems derived from human sources, or recombinant human cytochrome P450 systems as an external activation system (Ku et al., 2007b).

47. There are a number of MoGAs by which a chemical may demonstrate an *in vitro* genotoxic effect that is either not relevant for humans (e.g. a rat specific metabolite) or has a threshold. The COM has reviewed the evidence for a number of threshold MoGAs and published a general guidance statement <https://www.gov.uk/government/publications/assessment-of-thresholds-for-in-vitro-mutagens>.

48. Threshold MoGAs can generally be attributable to non-DNA interactions or an overload of normal cellular physiology. In such cases a No Observed Effect Concentration (NOEC) can be determined and may be useful in evaluating risk. Investigations of a threshold-based MoGA need to be designed on a case-by-case basis and can be complex to interpret (Kirkland et al., 2007a).

49. There has been considerable debate regarding the highest concentration that should be used routinely in mammalian cell assays. The ICH has stated the maximum concentration tested for human pharmaceuticals should be 1mM (or 5000 µgm/L) in mammalian cell genotoxicity assays (ICH, 2012) which would have the effect of reducing the number of misleading positive results by avoiding the excessive concentrations where the cellular defence mechanisms might be overwhelmed (ICH, 2012). However, a reduction to 1mM is not consistent with the OECD recommendation for a top concentration of 10mM (or 2000 ug/mL) in mammalian cell genotoxicity assays (OECD, 2016a,c,d,e). Morita et al. (2014) showed that the reduction in the top concentration from 5000 to 2000 µgm/L for mammalian cell tests had no impact on sensitivity or specificity of *in vitro* chromosomal aberration tests. Another analysis of published data for the top concentration in mammalian cell genotoxicity tests identified a small number of carcinogens that (according to the publications) would not be detected in any part of a three test *in vitro* genotoxicity test battery (consisting of the Ames, mouse lymphoma and *in vitro* chromosomal aberration (CA) tests) if the testing concentration limit for mammalian cell assays were reduced from 10mM to 1mM (Parry et al., 2010). A further investigation of these carcinogens found that some positive results at concentrations above 1mM were not reproducible (i.e. they were not

genotoxic in mammalian cells under current OECD guideline protocols) and others were positive at concentrations below 1mM, particularly when continuous treatments in the absence of S-9 (not included in the original publications) were conducted. A new upper limit for mammalian cells tests of 1mM or 500 µg/ml (whichever is higher) has been proposed as sufficient to detect all genotoxic carcinogens that are negative in the Ames test (Kirkland and Fowler, 2010). Several international organisations have updated their guidance regarding upper limit selection (e.g ICH, 2012, OECD, 2016a,c,d,e; Galloway et al., 2011) although currently no international consensus has been reached. Precipitation of the chemical in the medium can also be used to define a maximal concentration or upper limit for testing. On balance, COM agreed that the maximum concentrations identified in ICH S2(R1) should be adopted.

50. There has also been considerable investigation of the role of excessive cytotoxicity in mammalian cells and choice of cell type as possible causes of misleading positive results (Blakey et al., 2008; Fellows et al., 2008b; Pfuhler, 2009; Pfuhler et al., 2011). The method used to assess cytotoxicity may affect the selection of the highest concentration tested and potentially the results obtained using mammalian cell genotoxicity assays (Fowler et al., 2010b; Kirkland et al., 2007c; Kirkland, 2010) and recommendations have been made to use cytotoxicity measures based on cell proliferation (Galloway, 2000). However, it is important to note that although excessive cytotoxicity may lead to misleading positive results, it may also result in misleading negative results when pronounced cell cycle delay occurs. A similar conclusion was reached at an international symposium on regulatory aspects of genotoxicity testing (Blakey et al., 2008).

51. Most cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems. These cell lines may often have impaired p53 function and altered DNA repair capacity (Kirkland et al., 2007c). There is some evidence that human lymphocytes are less susceptible to misleading positives than the rodent cell lines currently used (e.g. Chinese Hamster Ovary (CHO), V79, Chinese hamster lung (CHL)). Other cell systems such as the human cell lines HepG2, TK6 and MCL5 cells and the reconstructed human skin models and HepaRG show promise for future use (Fowler et al., 2010a; Kirkland et al., 2007c; Le Hegarat, 2010).

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

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- there is no doubt as to the quality of the study design and the conduct of the test,
- the spacing and range of test chemical concentrations rule out missing a positive response,
- sufficient treatment conditions and sampling times have been used.

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

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

55. 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 (Hayashi et al., 2011). Advice has been published on approaches to collecting historical control data. Ideally data should be reported in terms of means and confidence intervals for the distribution of baseline genotoxic effects rather than observed ranges where outliers can have a disproportionate effect. The dataset should be updated regularly and should be as large as possible. Negative historical control data should have been generated using a fixed testing protocol unless it can be demonstrated that changes in protocol do not impact on the range of values reported in studies (Hayashi et al., 2011).

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

### Discussion of Stage 1 Strategy: Specific Core Tests

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

57. The most widely used *in vitro* mutagenicity test is the bacterial reverse mutation assay for gene mutations developed by Ames and his colleagues using *Salmonella typhimurium* (Gatehouse et al., 1994). The very extensive database available for this assay justifies its inclusion in any initial genotoxicity testing for mutagenic hazard. Several strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the validated strains being TA1535, TA1537 (or TA97 or TA97a), TA98 and TA100. These strains detect effects at G-C-rich sites. To detect certain oxidising mutagens or hydrazines, that produce effects at A-T-rich sites, an additional strain such as TA102 or a repair-deficient *Escherichia coli* strain (WP2uvrA or WP2uvrA (pKM101)) should be included. In order to detect cross-linking agents, it may be preferable to include TA102 or to add a repair proficient *Escherichia coli* strain (WP2 or WP2 (pKM101)). Testing should be carried out both in the presence and absence of an appropriate exogenous metabolic activation system such as induced rat liver S-9. Both plate-incorporation and pre-incubation methods are widely used, and either is acceptable in all test guidelines.

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

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

59. The COM recommended in 2000 that equivalent information on clastogenicity could be obtained from the MNvit compared with CA testing in mammalian cells (metaphase analysis) but that aneuploidy could be more easily detected by MNvit. This has since been confirmed in a collaborative trial (Lorge et al., 2006). The COM was aware in 2000 of the ongoing protocol developments and validation of this assay but noted that development of an OECD guideline would take some time. Since 2000 there have been extensive and authoritative investigations of the utility of the *in vitro* micronucleus assay, and a European Centre for the Validation of Alternative Methods (ECVAM) retrospective validation study concluded that the MNvit is reliable and can be used as an alternative to the *in vitro* CA for the assessment of clastogenicity and has the benefit of more easily detecting aneuploidy (Corvi et al., 2008). OECD guideline 487 has now been adopted, with the latest revision in 2016 (OECD, 2016a).



Many current *in vitro* genotoxicity testing strategies recommend that the micronucleus assay and metaphase analysis can be considered as equivalent in the detection of clastogens (Cimino, 2006; Eastmond et al., 2009). However the detection of aneugens in the metaphase test requires non-standard approaches and the COM recommends the MNvit assay as the first choice test for clastogenicity and aneuploidy detection.

60. The MNvit can be carried out in the absence or presence of cytochalasin B, which is used to block cell division and generate binucleate cells (cytokinesis block methodology (CBMN)). The advantage of using cytochalasin B is that it allows clear identification that treated and control cells have divided *in vitro* during or after treatment and provides a simple assessment of cell proliferation. In general, the use of cytochalasin B has no impact on the sensitivity of the test results (Garriott et al., 2002; Lorge et al., 2006; Oliver et al., 2006; Wakata et al., 2006), however this is not the case for nanoparticles as discussed in the document 'Test Guidance Strategies for Manufactured Nanomaterials' (COM, 2019). The target population in the presence of cytochalasin B are the binucleate cells (because it is clear they have divided); however scoring of both mononucleated and binucleated cells can be useful for the detection of aneugens (Lorge et al., 2006; Wakata et al., 2006). In the absence of cytochalasin B, it is essential to have evidence that cells have divided.

61. There have been major international collaborative investigations to develop the protocol (Aardema et al., 2006; Clare et al., 2006; Garriott et al., 2002; Kirsch-Volders et al., 2003; Lorge et al., 2006; Phelps et al., 2002), to provide information on the performance of this assay using different cell lines (Oliver et al., 2006; Pfuhrer et al., 2011; Wakata et al., 2006), and to investigate the most appropriate methods for measuring cytotoxicity (Fellows et al., 2008a; Fowler et al., 2012b; Kirkland, 2010; Lorge et al., 2008). There have also been initial studies to evaluate a flow cytometric approach to the micronucleus assay (Bryce et al., 2008a; Bryce et al., 2007; Laingam et al., 2008). The MNvit can be performed using most mammalian cell lines used in genotoxicity testing (Lorge et al., 2006). However there is evidence that rodent cell lines with compromised p53 activity such as V79, CHO and CHL cells can give more misleading positive results than cell lines proficient for p53 activity such as TK6 and human lymphocytes (Fowler et al., 2012a). Overall the COM's preference is for human lymphocytes which have a number of advantages over cell lines (e.g. normal diploid primary human cells with some protection against oxidative damage when whole blood cultures are used). If cell lines are used, it is important that the impact of potential genetic drift of the cells cultured is understood (Kirkland et al., 2007c). One particular area of protocol development which has been subject to considerable investigation is

the most appropriate method(s) for estimating cytotoxicity in MNvit tests (Fellows et al., 2008a; Kirkland, 2010; Lorge et al., 2008). It has been suggested that using relative cell counts (RCC) may underestimate cytotoxicity and lead to potentially misleading positive results (Fowler et al., 2012b). 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. Consensus recommendations embedded in the OECD guideline 487 indicate that the target range for cytotoxicity in the MNvit is  $55 \pm 5\%$ .

62. The *in vitro* micronucleus assay in combination with the CB methodology and with pancentromeric or chromosome specific centromeric probes fluorescence *in situ* hybridisation (FISH) provides a sensitive assessment of cell proliferation and allows discrimination between chromosome breaks, chromosome loss (using pancentromeric or anti-kinetochore antibodies) and chromosome non-disjunction and polyploidy (using whole centromere probes) (Kirsch-Volders et al., 2002). It is therefore a useful model for assessing mode of action (Parry, 2006).

63. Binucleate cells obtained with the CBMN will usually be needed for determination of non-disjunction of chromosomes between daughter nuclei. Fenech has proposed that the CBMN assay can be further modified to provide comprehensive information on nucleoplasmic bridges (NPBs). This may provide information on chromosome rearrangements or telomere end fusions, and nuclear buds (NBUDs) which may provide information on gene amplification (Fenech, 2006, 2007). Fenech proposed that the comprehensive CBMN assay should be considered as a 'cytome' method for measuring chromosomal instability and altered cellular viability (Fenech, 2006). The 'cytome' method is complex and requires large amounts of blood and considerable technical skill. It is currently not suitable for routine testing of chemicals for genotoxicity but may provide useful information on MoGA.

64. The flow-cytometry-based micronucleus assay (FCMMN) has the potential for increased reproducibility and decreased turnaround time for the micronucleus test (Laingam et al., 2008; Avlasevich et al., 2011). However the potential still exists for misleading positive results from cell processing or from chemical induced apoptosis and necrosis (Laingam et al., 2008; Avlasevich et al., 2011). Approaches to overcoming potential misleading positive results have included: the use of differential staining of micronuclei (MN) and necrotic and apoptotic cells (Bryce et al., 2008a; Bryce et al., 2007), the use of electronic gating procedures and the use of concurrent assessment of cytotoxicity such as the inclusion of 'counting beads' to MN assay



cultures which improve the calculation of relative cell survival (Laingam et al., 2008; Avlasevich et al., 2011). The FCMMN assay has also been adapted to cell lines which attach to solid surfaces (Bryce et al., 2010). The COM recognises the ongoing validation of the *in vitro* FCMMN assay which is important before it can be used for regulatory submissions. A separate approach to automation of the CBMN assay involves automated image analysis using Giemsa stained slides (Decordier et al., 2009; Avlasevich et al., 2011) which may be useful with appropriate validation.

65. An interlaboratory evaluation of the MultiFlowVR DNA Damage kit— p53, γH2AX, Phospho-Histone H3 has been described by Bryce et al. (2017). This is a multiplexed *in vitro* genotoxicity assay based on flow cytometric analysis in which detergent-liberated nuclei are simultaneously stained with propidium iodide and labelled with fluorescent antibodies against p53, γH2AX, and phospho-histone H3.

66. From seven laboratories assessing chemicals representing clastogens, aneugens and non-genotoxicants, with analysis based on global evaluation factors and using a multinomial logistic regression, assay sensitivity, specificity and concordance in relation to a priori MoA grouping were 92%. The authors suggest that the two distinct analysis strategies utilised can be used to rapidly and reliably predict a genotoxic MoA for new chemicals.

### Discussion Stage 1: Non-Core Tests

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

67. The *in vitro* CA assay in mammalian cells has been widely used in genotoxicity testing for many decades and provides information on chromatid and chromosome breaks, deletions and re-arrangements that are indicative of damage associated with adverse health outcomes. Only limited information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (Aardema et al., 1998). The COM notes that polyploidy may not be a reliable indicator for aneugenicity and may result from a number of different genetic changes (Galloway, 2000; Mitchell et al., 1995). It is possible to adapt the chromosome aberration assay to include the use of chromosome specific centromeric probes with fluorescence *in situ* hybridisation (FISH) to assess the potential for aneuploidy (Maierhofer et al., 2002). An IWGT report (Galloway, 2000) has concluded that the preferred measure of cytotoxicity in the CA test should be one based on cell proliferation (e.g. relative population doubling or relative increase in cell counts) compared to negative control cultures rather than simple cell counts. On balance it is

considered preferable to use the *in vitro* micronucleus test for the initial assessment of clastogenic and aneugenic potential. The latest revision of the OECD test guideline (OECD, 2016c) utilises a maximum test concentration corresponding to 10 mM (or 2 mg/mL) which is in-line with the revised MNvit assay (OECD, 2016a).

### *In Vitro Mouse Lymphoma Assay for Gene Mutation and Clastogenicity*

68. The COM reaffirms the view stated in the 1989, 2000 and 2011 guidance, that the most appropriate *in vitro* mammalian cell gene mutation test is the mouse lymphoma assay.

69. Since 2000, there has been considerable development of suitable protocols, negative solvent control data, criteria to define an acceptable positive control response and the use of the Global Evaluation Factor (GEF) and statistical analysis for interpretation of test results (Clements, 2000; Kirkland et al., 2007c; Moore et al., 2007; Moore et al., 2003; Moore et al., 2006). Many of the published studies were undertaken by the US NTP and a re-evaluation of these results shows many of the studies to be uninterpretable or the outcomes to be equivocal (Schisler et al., 2018). The latest revision by OECD was published in 2016 (OECD, 2016d). Some authors have reported that the mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity, information on recombination, deletion and aneuploidy (Ogawa et al., 2009; Sofuni, 1996; Wang et al., 2009). However, this has been contested from results showing that none of 7 reference aneugens were reliably detected at acceptable levels of cytotoxicity (Fellows et al., 2011b). It is possible that aneuploidy in these cells could be a secondary effect of chromosomal rearrangement. However, the COM considers that this assay does not detect aneugens.

### *In Vitro HPRT assays for Gene Mutation*

70. An *in vitro* cell mutation assay which uses forward mutation in the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene to assess mutations has been developed in several cell lines, principally CHO cells (Li, 1988). It is described in the OECD 476 guideline (OECD, 2016e). The COM have previously considered the power of this assay and it was concluded that  $10^7$  surviving cells are required for a valid test, (<http://webarchive.nationalarchives.gov.uk/20140506144831/http://www.iacom.org.uk/meetings/02.10.2003.htm>), providing sufficient numbers of cells to undergo between 10 and 100 spontaneous mutations. Thus, certain mammalian cell gene mutation protocols that have been widely used, particularly some involving CHO cells, are considered to be insufficiently sensitive for the identification of mutagens, predominantly on statistical grounds (UKEMS, 1989).

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

71. As discussed in para 44, a number of research groups have developed genotoxicity assays based on MN measurement using commercial sources of human reconstructed skin (such as Episkin® and EpiDerm™) (Chapman et al., 2014; Curren et al., 2006; Flamand et al., 2006; Hu et al., 2009; Mun et al., 2009; Roy et al., 2016) or a co-culture technique involving reconstructed skin and mouse lymphoma L5178Y cells (Flamand et al., 2006). Measurement of DNA damage using the comet assay in reconstructed skin has also been reported (Pfuhler et al., 2011; Reisinger et al., 2018). The primary purpose in developing genotoxicity tests using reconstructed skin has been to supplement genotoxicity data-packages for cosmetic chemicals where no *in vivo* genotoxicity tests are permitted. A tiered approach to testing cosmetic ingredients for genotoxicity has been published (Pfuhler et al., 2010).

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

72. The *in vitro* alkaline comet assay for DNA damage has been proposed as an alternative to clastogenicity assessment in mammalian cells since cell proliferation is not needed, therefore any cell type can be used (Hartmann et al., 2001; Witt et al., 2007). The alkaline comet assay detects a wide range of genetic damage including single and double strand breaks, repair induced breaks, alkali labile lesions and abasic sites. There is evidence that the *in vitro* comet assay can be modified to detect DNA cross-linking agents (Spanswick et al., 2010). The comet-FISH assay has been developed to provide information on site specific DNA strand breaks (Glei et al., 2009; Rapp et al., 2000; Santos et al., 1997). There is evidence that the *in vivo* comet assay can detect chemicals that induce gene mutations *in vitro* (Dertinger et al., 2010; Kirkland and Speit, 2008). Extrapolation from this suggests that the *in vitro* comet assay can also detect chemicals that induce gene mutations and this capability has been demonstrated (Dertinger et al., 2010). However, it is not recommended as a routine replacement for gene mutation tests *in vitro*. Thus, the comet assay measures DNA damage irrespective of genotoxic end-point, with the exception of aneuploidy. A positive comet assay result may be due to repairable DNA damage or lesions which lead to cell death and not necessarily mutations or MN. Negative results from an Ames test and MNvit would reduce the level of concern associated with positive results from an *in vitro* comet assay. Thus, the *in vitro* comet assay can serve as a useful adjunct to the recommended core-tests, especially in instances where *in vivo* testing is not permitted such as in cosmetic testing.

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

73. The COM recommendations for Stage 1 testing remain the same as in the 2011 guidelines, namely that the three key endpoints of gene mutation, clastogenicity and aneuploidy can be detected by using two core *in vitro* tests. Tests should be undertaken according to the best international guidance available to avoid misleading positive or negative results. Data should be interpreted using appropriate statistical analysis and use of historical negative control data. The COM confirms the need to understand MoGA in order to derive conclusions regarding the biological importance of results. Data on MoGA are important in elucidating whether genotoxicity tests give misleading negative or positive results, and also to aid decisions with regard to devising a strategy for Stage 2 *in vivo* genotoxicity testing. There is a particular need to understand MoGA for chemicals which cannot be subjected to *in vivo* genotoxicity tests (e.g. cosmetics). In this particular instance some useful additional information on genotoxicity may be provided by undertaking further testing, for example using *in vitro* mammalian cell gene mutation assays or *in vitro* MN and comet tests using reconstructed human skin. The recommended two core genotoxicity tests in Stage 1 are the Ames test and MNvit. These recommended assays, when combined, provide sufficient information for the genotoxicity assessment of most chemicals and provide high sensitivity for the identification of rodent carcinogens and *in vivo* genotoxicants, and reduce the risk of misleading positive results when compared with a battery containing more than one mammalian cell test. Results from non-core tests described in this document may provide useful additional information on *in vitro* mutagenic hazards on a case-by-case basis. In most instances misleading negative *in vitro* results are due to inadequate exogenous metabolic activation (Ku et al., 2007b). However, some regulatory authorities may require an *in vivo* genotoxicity test where high, or moderate and prolonged, levels of exposure are expected (e.g. most human medicines) in order to provide additional reassurance even when Stage 1 tests have given negative results. If a chemical is considered on the basis of Stage 1 test results to have *in vitro* mutagenic potential but has not been tested *in vivo*, the COM considers it prudent to assume that the chemical may have *in vivo* mutagenic potential.

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

#### Overview of Strategy

74. Stage 2 of the testing strategy involves an assessment of genotoxic activity *in vivo* in somatic tissues and in germ cells (when there is a need for the assessment of heritable effects and/or information on hazard classification of mutagens) (see Figure

3). The *in vivo* genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to address aspects of *in vivo* mutagenicity, for example;

- Investigation of key end point(s) identified in Stage 1,
- Investigation of genotoxicity in tumour target tissue(s),
- Investigation of potential for germ cell genotoxicity,
- Investigation of *in vivo* genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure.
- Investigation of genotoxicity in site of contact tissues.

75. It is thus possible for there to be one or more separate Stage 2 strategies designed to assess the above objectives for a particular test chemical. This rationale leads to different approaches from those advocated by the COM in 2011 where the weight of available evidence suggested that the *in vivo* bone marrow (or peripheral blood) micronucleus assay or bone marrow clastogenicity assay in rodents was the preferred first test in almost all cases. The exception was for direct acting DNA reactive mutagens where a site of contact test was the preferred first test. There was a preference in the 2000 COM guidance for the rat liver UDS assay as a second tissue *in vivo* test, which was selected primarily to provide reassurance of absence of *in vivo* genotoxicity when positive results had been obtained *in vitro* but negative results were obtained in an *in vivo* BMMN 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). The revised *in vivo* Stage 2 strategy based on the selection of tests to provide information on one or more specific aspects such as species and/or tissue genotoxicity combined with investigation of particular genotoxic end points and modes of genotoxic action does not necessarily lead to the selection of the rodent BMMN test as the first assay. Furthermore, the rat liver UDS assay is no longer recommended as a second assay (EFSA, 2017 - discussed in para 96). A table of *in vivo* genotoxicity tests and end-points is provided in Annex 2.

76. Other factors that should be considered when determining an *in vivo* genotoxicity testing strategy include whether the testing strategy can be integrated into other regulatory toxicity tests (such as subacute or subchronic toxicity studies). A Consideration needs to be given to the nature of the chemical (including physico-chemical properties), the results obtained from *in vitro* genotoxicity tests and the available information on the toxicokinetic and metabolic profile of the chemical (for example when selecting most appropriate species, tissue and end point). The routes

of exposure in animal studies should be appropriate to ensure that the chemical reaches the target tissue. Routes unlikely to give rise to significant absorption in the test animal should therefore be avoided. Unless systemic exposure can be confirmed from other toxicological studies, or evident toxicity in the target organ is seen, confirmatory toxicokinetic studies to measure blood or tissue exposure as appropriate should be undertaken to accompany all *in vivo* genotoxicity studies to assess the adequacy of any negative results obtained (Hardy et al., 2017).

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

- use of one sex only (if supported by metabolism data or other data indicating equivalence),
- reduced numbers of sampling times for micronucleus and CA assays when repeat dosing is performed,
- combining micronucleus and comet assays into a single acute test employing repeated administrations of test chemical; integration of micronucleus and comet end points into repeat-dose toxicity (including transgenic mutation) studies, although it should be noted although it should be noted that the comet assay is difficult without using satellite groups (Bowen et al., 2010; Bowen and Beevers, 2011; Pfuhler et al., 2009; Vasquez, 2010)

78. It should also be possible to omit the concurrent positive control administrations in micronucleus, CA and transgenic rodent mutation assays (but not for the comet assay) where the test facility has appropriate historical positive control data (Pfuhler et al., 2009) as long as positive control slides or tissues from positive control treated transgenic rodents “banked” from previous treatments and coded in with the experimental samples, are included to demonstrate technical proficiency.

79. The toxic properties of test chemicals (such as acute toxicity, subchronic toxicity (including target organ effects), irritancy/corrosivity in contact with skin or mucous membranes), toxicokinetic and metabolism data will influence the choice of route of administration and the highest dose level achievable in *in vivo* mutagenicity tests. Dose selection for *in vivo* genotoxicity testing requires estimation of the maximum tolerated dose, consideration of tissue-specific effects and in some instances (as discussed in paragraph 76), appropriate toxicokinetic data or toxicity data

in the target tissue from other studies, to support tissue exposure to the chemicals and/or metabolites.

80. The approach outlined to Stage 2 in Figure 3 takes account of evidence to suggest that *in vivo* comet and rodent transgenic mutation assays have better sensitivity and specificity for the identification of rodent carcinogens compared with the rat liver UDS test, particularly for carcinogens that are negative in the *in vivo* micronucleus test (Kirkland and Speit, 2008). The initial *in vivo* genotoxicity testing strategy should therefore involve selection of one or more of the core Stage 2 tests in rodents; namely, micronucleus tests (accompanied by specific assays for aneuploidy if necessary), the transgenic gene mutation tests, or comet DNA damage assays in rodents. It is acceptable to undertake one *in vivo* genotoxicity test to investigate a specific end point identified from Stage 1 *in vitro* genotoxicity tests. In some instances there may be a need to investigate more than one end point before reaching a full conclusion on *in vivo* genotoxic potential.

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

82. In addition, an *in vivo* genotoxicity test may give positive results for chemicals which only act *in vivo*; experience though, has shown that such chemicals are rare {Tweats, 2007a,b}. Such agents induce some kinase inhibitors, glucocorticoid receptor antagonists (Hayes et al., 2013) and long-acting beta-2-agonists (Ponten et al., 2013). In some instances positive results might be obtained from *in vitro* genotoxicity tests that are adapted to evaluate specific characteristics of the test chemical; for example, by the use of modified or non-standard exogenous metabolising fractions (Muller et al., 2003).

83. Positive results in any Stage 2 genotoxicity test should be assessed for an indication of a MoGA and for evidence which may suggest a threshold of effect or



irrelevant positive responses. The COM has previously discussed the relevance of high-dose only positives and recognises that these results may be secondary to non-genotoxic effects rather than being a genotoxic effect of the compound (<http://webarchive.nationalarchives.gov.uk/20140506144902/http://www.iacom.org.uk/statements/COM03S5.htm>).

84. Examples of such modes of action in micronucleus tests, include hypothermia or hyperthermia in rodents and compound induced increases in cell division of bone marrow erythroblasts (Blakey et al., 2008; Shuey et al., 2007; Tweats et al., 2007a). If the conclusion is reached that a MoGA occurs then the chemical should be considered as an *in vivo* mutagen. MoGA data will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The COM has published guidance on possible threshold modes of genotoxicity which can include; i) involvement of non-DNA targets, (e.g. aneugen inhibition of microtubules), ii) the contribution of protective mechanisms (e.g. repair of DNA adducts formed from many low molecular weight alkylating agents) and, iii) overload of detoxication pathways (e.g. paracetamol)

([https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/315698/assessment\\_of\\_threshold\\_for\\_in\\_vivo\\_mutagens.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/315698/assessment_of_threshold_for_in_vivo_mutagens.pdf)).

85. Supplementary *in vivo* tests should be undertaken if the results of the core *in vivo* genotoxicity test(s) (para 80 and Figure 3) give equivocal results or if there is a need to investigate specific mutagenic endpoints, tumour target organs, or the potential for heritable effects. This may involve repeating all or aspects of the initial Stage 2 testing strategy, or performing supplementary investigations (e.g. mode of action investigations, such as DNA adducts or more specific germ cell testing) to investigate aspects of the genotoxicity of the test 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, toxicological effects and metabolism of the chemical, should be considered.

86. The development of testing strategies for germ cell mutagens is a rapidly evolving field. A summary of test methodologies that are currently under development and/or validation are outlined in the document 'Test Strategies for Germ Cell Mutagens' (COM, 2019).

87. One aspect of the approach to testing outlined in Figure 3 is that hazard characterisation of germ cell genotoxicity can be included in the initial *in vivo* genotoxicity testing strategy if considered necessary. This is because there are multi



tissue *in vivo* genotoxicity assays (e.g. transgenic rodent mutation assays and comet assay, though it should be noted that the standard comet assay is not useful for a meaningful assessment of genotoxicity in mature sperm) which can also be used if a need to evaluate germ cell genotoxicity has been established. However, there are uncertainties around optimal sampling times for germ cells and further evaluation of these assays for this purpose is recommended. Additionally, germ cell mutation assays might be valuable on a case-by-case basis to provide information on heritable mutagenic effects, but these would form part of a supplementary *in vivo* genotoxicity testing strategy, if considered appropriate.

88. The COM reaffirms that a chemical considered a positive *in vivo* somatic cell mutagen should also be considered as a possible germ cell mutagen unless data can be provided to the contrary. The position held previously, that most if not all germ cell mutagens are also genotoxic in somatic cells still holds true. It has been noted that there are some rare examples (e.g. sodium orthovanadate, (Attia et al., 2005) where the mouse bone marrow micronucleus assay does not predict germ cell genotoxicity. However, the data on such compounds are conflicting and it is not known, for example, whether somatic mutations or DNA strand breaks would have been identified if other test systems (e.g. transgenic assays and the comet assay) had been used and other tissues sampled (Attia et al., 2005; Ciranni et al., 1995; Witt et al., 2003). It is possible these rare examples may relate to cellular targets in germ cells that are not present in the bone marrow (e.g. different proteins in chromatin structure and processes involved in meiosis). However, induction of other genotoxic effects and in other tissues cannot be excluded. There are also examples of germ cell mutagens which affect specific stages of gametogenesis in males (Adler, 2008) and where there are differences between male and female germ cell genotoxicity (Bishop, 2003).

89. It is plausible that other targets during the process of meiotic cell division may be unique to germ cells but not necessarily identical in both sexes (Pacchierotti et al., 2007). The COM evaluated recent advances in germ cell mutagenicity testing and some theories and hypotheses regarding human germ cell mutagenesis. It was concluded that further validation work was needed before newly developed germ cell assays, such as the sperm comet assay, could be incorporated into general genotoxicity testing. There are a number of methodological difficulties involved in the analysis of germ cells compared to somatic cells (e.g. germ cell DNA extraction) and the importance of good study design was highlighted. The COM concluded that it is not known whether unique germ cell mutagens exist (i.e. chemicals that are germ cell mutagens but not somatic cell mutagens), but that this is partially because of the

underutilisation of the currently accepted tests for assessing germ cell mutagenicity and a lack of investigations examining the possibility

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

90. There are many publications debating *in vivo* genotoxicity testing strategies. For example, the GUM (German speaking section of the European Environmental Mutagen Society) recommended a single study using a combined analysis for MN and comet induction in selected tissues (Pfuhler et al., 2007), while the World Health Organization/International Programme on Chemical Safety (WHO/IPCS) recommended cytogenetics (bone marrow) or gene mutation or alternative tests as defined by genotoxic end-point, chemical class and reactivity (with consideration of factors such as bioavailability and metabolism) (Eastmond et al., 2009). The *in vivo* genotoxicity testing strategy recommended by the COM acknowledges there can be a variety of reasons for undertaking *in vivo* genotoxicity tests and it is important to identify clearly the objective of the study and the critical aspects of *in vivo* genotoxicity to be addressed (as set out in the Overview of Stage 2 strategy; paras 74-86) in order to develop a strategy accordingly, rather than simply specify preferred first and second tests. There are less data on the performance of *in vivo* genotoxicity assays for prediction of rodent carcinogenicity compared to data on the performance of *in vitro* genotoxicity tests. Transgenic rodent mutation assays and the *in vivo* micronucleus assay have been shown to exhibit complementarity regarding prediction of rodent carcinogenicity, consistent with the assessment of different mutagenic end-points by these two assays (Morita et al., 2016). Transgenic rodent mutation assays were usually positive for those carcinogens which were positive in *in vitro* gene mutation tests in bacteria whilst the *in vivo* MN assay had greater predictivity for carcinogens positive in the *in vitro* metaphase analysis in mammalian cells (Lambert et al., 2005). Thus genotoxic end-point and MoGA analysis of *in vitro* mutagenic activity is of considerable importance in helping to develop an initial *in vivo* genotoxicity testing strategy. The COM recommends that the initial *in vivo* genotoxicity testing strategy should be based on one or more tests selected from a relatively limited number of *in vivo* genotoxicity tests that have been specifically designed to provide the optimum amount of information on *in vivo* mutagenic potential of the test chemical.

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

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

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

*OR Rodent Bone Marrow CA Assay for Clastogenicity*

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

93. Proposals have been published to incorporate micronucleus assays into routine rodent 28 day subacute toxicity studies which have demonstrated the feasibility of such an approach (Hamada et al., 2001; Krishna et al., 1998; Madrigal-Bujaidar et al., 2008). The development of a simultaneous liver and peripheral blood micronucleus assay in adult rats has also been reported (Suzuki et al., 2005b). The evidence from one evaluation of micronucleus tests conducted on samples from short-term, subchronic

and from a few chronic studies in mice has been published. In mice, MN in polychromatic erythrocytes represent DNA damage occurring in the last 72h, whilst MN in normochromatic erythrocytes represent average damage during the 30 day period prior to sampling (Witt et al., 2000).

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

93. There has been a significant increase in the number of studies undertaken with transgenic rodent mutation assays published since the COM guidance in 2000. These have been comprehensively reviewed (Lambert et al., 2005; Morita et al., 2016; OECD, 2009). There are sufficient data to assess the performance of the Muta<sup>TM</sup> mouse, BigBlue<sup>®</sup> mouse and rat (including use of  $\lambda$  cII transgene), *LacZ* plasmid mouse, and the *gpt* delta mouse models, although it is noted that the *gpt* models are not widely used and are less well validated. The transgenic rodent mutation assays can be used to assess gene mutations in a wide range of rodent tissues (including germ cells) using all routes of administration (Lambert et al., 2005) and is particularly valuable when investigating gene mutation as the genotoxic endpoint. Transgenic rodent mutation assays have been reported to produce data that are generally compatible with the mouse specific locus test for germ line mutagens (Singer et al., 2006). However, some uncertainties remain with regards to the optimal sampling time for germ cells (Yauk et al., 2015)

Transgenic rodent mutation assays are considered particularly useful for *in vivo* site-of-contact mutagen assessment (Dean et al., 1999). Guidance on appropriate approaches to protocol development have been published by the IWGT (Thybaud et al., 2003). Molecular sequencing 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 transgenic rodent mutation assays although the available data suggests the best positive and negative predictivity was obtained using results from *in vitro* bacterial mutagenicity tests and *in vivo* transgenic rodent mutation assays (Lambert et al., 2005). There is an ongoing need to consider and validate the optimal protocol when using transgenic gene mutation tests with tissues with a slow turnover. The OECD published a Detailed Review Paper (DRP) on Transgenic Rodent Gene Mutation Assays in 2009 and recommended the

development of an OECD guideline (OECD, 2009). An OECD guideline was adopted in July 2011 and revised in 2013 (OECD, 2013) .

### *Rodent Comet Assay for DNA Damage*

94. The *in vivo* comet assay detects a wide spectrum of DNA damage including repairable DNA damage. An overview of the types of genetic lesions detected is given above in paragraph 59. The *in vivo* comet assay can detect chemicals that induce gene mutations and has produced positive results for nearly 90% of rodent carcinogens not detected by the rodent BMMN assay (Kirkland and Speit, 2008). It also shows high sensitivity when compared with TGR results in liver and the GI tract, and high sensitivity at detecting bacterial mutagens and mutagenic carcinogens (GTTC and IWGT reports). There have been significant developments with regard to the conduct of the *in vivo* alkaline comet assay since 2011 (Burlinson et al., 2007; Brendler-Schwaab et al., 2005; Hartmann et al., 2004). This assay can be used for elucidating positive *in vitro* genotoxicity findings and to evaluate genotoxicity in target organs of toxicity (Hartmann et al., 2004), however, it would not be an appropriate follow-up for a chemical causing aneuploidy *in vitro*. The comet assay can be applied to a wide range of species and in many tissues including site-of-contact tissues. In the absence of data indicating particular tissues of interest (e.g. toxic findings or tissue accumulation seen in other studies), comet analysis of the stomach/duodenum (to detect site of contact effects), and liver (to detect genotoxic metabolites) should be studied. Extensive validation exercises and assay evaluations underpinned the development of a standardised protocol (Burlinson et al., 2007; Speit et al., 2015; Uno et al., 2015). An OECD Guideline was adopted in July 2016 (OECD, 2016b). However, agreement on a method for a germ cell comet assay was not achieved and further protocol modifications and validation studies are considered necessary (Speit et al., 2009).

95. The Committee considers that the *in vivo* comet assay has appropriate sensitivity to detect chemicals which induce both gene mutations and/or clastogenicity. Thus the *in vivo* comet assay is recommended as a core test in the initial *in vivo* genotoxicity testing strategy to assess DNA damage in multiple somatic tissues in a single study. It is possible to include the comet assay within other *in vivo* genotoxicity tests (Vasquez, 2010) or within standard subacute or subchronic regulatory toxicity tests (Rothfuss et al., 2010), although the logistics of timing the final doses prior to tissue sampling must be carefully considered (Speit et al., 2015).

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

96. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver (Kennelly et al., 1993). The endpoint measured is indicative of DNA damage and subsequent repair in liver cells. The COM consideration of this assay and published evaluations now suggest it is less sensitive than the *in vivo* comet assay with regard to identification of genotoxicity in the liver. 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; Speit et al., 2015). Based on these analyses, EFSA concluded that the UDS assay was of limited usefulness in genotoxicity testing strategies, being only suitable for the detection of chemicals causing damage in the liver, and with a lower predictive value than the TGR and comet assays in detecting chemicals which cause gene mutations. For existing datasets, where the UDS assay has been used as a follow up to positive *in vitro* gene mutation findings, a UDS study is considered adequate only for positive results (Hardy et al., 2017). The COM agree with this opinion and recommend use of the comet assay rather than rodent liver UDS in order to assess potential for DNA damage *in vivo*.

### Discussion of Stage 2-Supplementary Tests.

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

98. A brief outline of these additional Stage 2 methods is given in Table 1 below. Reference is also made in Table 1 to a number of tests for heritable genotoxic effects but it is noted that these tests, which involve the use of many animals and demand a

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high level of expertise, are comparatively rarely used. The COM is aware that there is the possibility that gender differences in germ cell mutagenesis may exist and this aspect may need to be considered on a case-by-case basis (Eichenlaub-Ritter et al., 2007). The conclusions of COM's evaluation of germ cell testing methods are provided in a summary document:

**Table 1      Supplementary in vivo genotoxicity tests**

Assay	Endpoint	Guidance	Main Attributes	Comments
<u>Investigations of DNA Adducts</u>				
<b><sup>32</sup>P-postlabelling</b>	DNA adducts	IWGT	Can be highly sensitive particularly with bulky adducts and if appropriate enrichment technique used.	Interpretation of results can be complex. Involves handling high-activity <sup>32</sup> P. (Phillips et al., 2000)
<b>Covalent binding to DNA</b>  A variety of methods can be used such as those involving radioactive decay measurements (eg. <sup>14</sup> C-) or isotope measurements (eg Accelerator Mass Spectrometry AMS)	DNA Adducts	IWGT	Some methods (AMS) are potentially very sensitive and can provide data on DNA binding at levels of exposure similar to low level environmental exposures	Uses radiolabelled compound (very small amounts (e.g. nanograms) in the case of AMS). Interpretation of results can be complicated (e.g. by non-specific binding). (Himmelstein et al., 2009)
<b>Supplementary investigations of germ cell mutagenicity</b>				
Analysis for clastogenicity/aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes	OECD	Can provide information on nature of effects in spermatogonia, spermatocytes and/or oocytes of mice or rats	Can provide useful information on MoGA. (Russo, 2000)
Spermatid micronucleus assay	Chromosomal aberrations and or lagging chromosomes	None available	Provides information of clastogenic and/or aneugenic effects in spermatocytes.	(Allen et al., 2000)
Dominant lethal assay	Chromosomal/gene mutations	OECD	Provides information on unstable chromosomal changes in gametes that lead to fetal death after fertilization and can determine stage(s) of gametogenesis affected	Little used. needs relatively large numbers of animals (Adler et al., 1994)
Mouse specific locus test	Gene mutations	EPA	Provides information on genetic changes transmitted to the first generation progeny as basis for estimation of induced mutation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on chromosomal changes transmitted to the first generation progeny as basis for estimation of induced translocation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Sperm Comet assay	Double strand breaks and/or apurinic sites in sperm head DNA	None available	Provides information on genetic instability in sperm	(Trivedi et al., 2010)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes	EPA	Provides information on induction of DNA lesions	(Sotomajor and Segal, 2000)



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

99. The *in vivo* genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to address aspects of *in vivo* mutagenicity, for example;

- Investigation of key end point(s) identified in Stage 1,
- Investigation of genotoxicity in tumour target tissue(s),
- Investigation of potential for germ cell genotoxicity,
- Investigation of *in vivo* genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure.
- Investigation of genotoxicity in site of contact tissues.

100. The recommended *in vivo* genotoxicity test(s) include micronucleus assay, bone marrow cytogenetics, comet assay in rodents and transgenic rodent mutation assay. In some instances there may be a need to undertake more than one *in vivo* test to perform an initial assessment of *in vivo* genotoxic potential (e.g. where endpoints cannot be assessed in one study and there is a need to investigate multiple end points before reaching conclusions on *in vivo* mutagenic potential). Multiple endpoints may be combined in a single study. If positive results are obtained it is important to consider the evidence for genotoxic mode of action and check the data for evidence of irrelevant positive results. Usually negative results obtained in a carefully selected *in vivo* test (possibly studying more than one endpoint and tissue) will be sufficient to address positive results found *in vitro*, provided that target tissue exposure is sufficient. However, a further test(s) may be needed if some of the genotoxic effects seen in Stage 1 *in vitro* tests had not been adequately studied *in vivo* (e.g. the chemical affects multiple mutagenic end-points), or other aspects of the genotoxic potential of the chemical had not been fully resolved (e.g. in the case where an investigation of heritable effects was required). If equivocal results are obtained, then supplementary testing may be needed. This may involve repeating some aspects of the recommended *in vitro* and/or *in vivo* genotoxicity tests, or performing additional investigations (e.g. MoGA investigations, such as DNA adducts and/or more detailed consideration of heritable effects). The supplementary *in vivo* genotoxicity testing strategy should be devised on a case-by-case basis. There is a need to select the most appropriate assay(s) on a case-by-case basis. All relevant factors such as results from previous tests, structural alerts and available information on toxicokinetics, tissue toxicity and metabolism of the chemical, should be considered. In the absence of appropriate germ

cell genotoxicity data, the COM considers it is reasonable to assume that all somatic cell mutagens have the potential to be germ cell mutagens.

### Possible Future Developments

101. The COM is aware that new assays and toxicogenomic approaches are under development which might be of value within genotoxicity testing. These include the detection of gene mutations at the endogenous phosphatidylinositol glycan complementation group A gene (*Pig-A*), a reporter gene in which mutations are currently detected in peripheral red blood cells of mammals (Bryce et al., 2008b; Dertinger et al., 2011; Miura et al., 2009). This assay has potential advantage of integration into regulatory toxicity tests (Dertinger et al., 2010; Khanal et al., 2018) and it is noted that *Pig-A* mutations increase with duration of dosing (Miura et al., 2009). The disadvantage of the assay is that, to date, only limited types of cells (red blood cells/ reticulocytes and male rat germ cells) have been used. It is anticipated that further developments and validation will lead to the development of an OECD Guideline.

102. The ToxTracker assay uses a series of reporter cell lines expressing biomarker genes selected to identify chemically induced DNA damage and oxidative stress (Hendriks et al., 2012; Hendriks et al., 2011). Whilst the assay presents an interesting approach to identifying MoGA, it is not currently considered to be a reliable genotoxicity test and is more suitable as a biomarker assay or in MoGA investigations.

103. Other potential tests include investigation of instability in expanded simple tandem repeats in male gametes and offspring to evaluate heritable mutations (Singer et al., 2006). The development of new high throughput assays for the assessment of germ line mutations and the quantification of risk from such data may provide opportunities to protect future generations from mutated DNA sequences. Developments within the field of toxicogenomics are also likely to provide new methods for investigating genotoxic mechanisms and informing on MoGA. The COM have reviewed data generated in this field several times during 2008 and 2009 up to the drafting of this guidance statement but currently conclude that the evidence does not support the routine use of toxicogenomic approaches as an adjunct to genotoxicity testing.

104. A workshop held by the ILSI-HESI (Health Effects Institute) IVGT (In Vitro Genetic Toxicity Testing) Project Committee reviewed 16 assays/technologies which

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were at various stages of development (defined as emerging to mature). The workshop highlighted emerging approaches to genotoxicity testing such as Enzyme-DNA films and DNA adductome studies (Lynch et al., 2011). More recently, ILSI-HESI considered 'next generation' testing strategies for genotoxicity including the use of QSAR modelling, MoGA assessments and their human relevance. The concept of quantitative assessment of genotoxicity data was also discussed (Dearfield et al., 2017). Quantitative approaches to the assessment of genotoxicity data was considered by COM in 2017-2018. Their conclusions were published in a statement (<https://www.gov.uk/government/publications/quantitative-approaches-to-the-assessment-of-genotoxicity-data>).

Annex 1. **Sensitivity and Specificity Data Considered by the COM**

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

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

107. The sensitivity and specificity data that have been reviewed by the COM are tabulated in Annex 1, Tables 1-3 (rounded to whole numbers). The list of tests for each stage is not necessarily exhaustive.

Annex 1 Table 1 QSAR data

Method	Sensitivity Identification of mutagens or rodent carcinogens	Specificity Identification of non- mutagens or rodent non-carcinogens	Comments/references
DEREK	No data reported	No data reported	Agreement with Ames positive 65% (416 compounds) (Cariello et al., 2002)
TOPKAT	No data reported	No data reported	Agreement with Ames positive 73% (416 compounds) (Cariello et al., 2002)
MDL QSAR	81%	76%	3338 compounds tested in bacterial mutagenicity tests (Contrera et al., 2005)
MultiCASE (MC4PC)	71% (bacterial) 63% (mouse lymphoma) 44% (clastogenicity in vitro) 53% (clastogenicity)	88% (bacterial) 74% (mouse lymphoma) 92% (clastogenicity in vitro) 75% (clastogenicity)	1485 compounds, bacterial. 328 compounds for mouse lymphoma. 556 compounds for clastogenicity (Matthews et al., 2006). 679 compounds (Roithfuss et al., 2006)
Toxtree (version 1.50)	74% (rodent carcinogenicity) 85% (bacterial mutagenicity)	64% (rodent carcinogenicity) 72% (bacterial mutagenicity)	878 chemicals with carcinogenicity data, 698 chemicals with mutagenicity data (Benigni and Bossa, 2008)

Annex 1 Table 2 Screening Tests

Method	Sensitivity <sup>a</sup>	Specificity <sup>b</sup>	Comments/references
HepG2 (cystatin, p53, Nrf2) luciferase reporter	85% (17/20 ECVAM list) 74% (bacterial mutagenicity) 45% (clastogenicity)	81% (34/42 ECVAM list) 80% (bacterial mutagenicity) 83% (clastogenicity)	62 ECVAM listed chemicals, 192 additional chemicals (Westerink et al., 2010)
Vitotox™ (bacterial SOS reporter assay for mutagenicity) RadarScreen (RAD54 reporter assay in yeast for clastogenicity)	70% bacterial mutagenicity (14/20 ECVAM list) 86% (bacterial mutagenicity).  70% clastogenicity (14/20 ECVAM list), 77% (clastogenicity)	93% (39/42 ECVAM list) 94% (bacterial mutagenicity)  83% clastogenicity (35/42 ECVAM list) 74% (clastogenicity)	62 ECVAM listed chemicals, 192 additional chemicals (Westerink et al., 2009)
GADD45a-GFP	18/20 ECVAM list of mammalian cell mutagens (90%)  95% for genotoxic carcinogens  63% (regulatory battery of Ames, CA/MNvit, or CA/MLA) 94% of genotoxicants    30% (in vitro genotoxicants) 30% (rodent carcinogens)	22/23 ECVAM list of mammalian cell non-mutagens (96%)  100% non-carcinogens 100% (regulatory battery of Ames, CA/MNT or CA/MLA) 83% non-genotoxicants   97% (in vitro genotoxicants) 88% (rodent carcinogens)	(Birrell et al., 2010)  75 compounds studied (Hastwell et al., 2006)  Validation data for 56 compounds requiring metabolic activation (Jagger et al., 2008)    57 <i>in vitro</i> genotoxicants 50 chemicals with rodent carcinogenicity data. (Olaharski et al., 2009)

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

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

**Annex 1 Table 3 Genotoxicity Tests (*in vitro*) in Relation to Rodent Carcinogenicity**

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

\*Positive results in at least one test

a: accurate prediction of rodent carcinogenicity

b: accurate prediction of rodent non-carcinogenicity



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

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



### Annex 3. Rationale for Selection of Ames Test and *In Vitro* Micronucleus Assay as The Two Principal *In Vitro* Assays. (Kirkland, 2011)

108. An evaluation of the use of *in vitro* genotoxicity tests to predict rodent carcinogens (557 chemicals evaluated) and *in vivo* genotoxicants (405 chemicals evaluated) was prepared and updated for the COM meeting in June 2010 (Paper MUT/2010/08).(<http://webarchive.nationalarchives.gov.uk/20140506144828/http://www.iacom.org.uk/meetings/documents/COMminsJune2010forinternet.pdf>). A two-test battery consisting of the Ames test plus *in vitro* micronucleus tests correctly identified 73% of rodent carcinogens. This is lower than in the published sensitivity analysis of Kirkland et al (Kirkland et al., 2005a), because in the current analysis the *in vitro* chromosomal aberration test was accepted as a surrogate for the *in vitro* micronucleus test where no data existed for the latter, as the concordance between the 2 tests for detection of clastogens is so high. Thus, the denominator used in the calculation of sensitivity for the current 2-test battery, by taking either *in vitro* micronucleus or *in vitro* chromosomal aberration results, is correspondingly larger than in the (Kirkland et al., 2005a) and lower sensitivity is reported. By adding the MLA as a third *in vitro* test, the sensitivity increased marginally to 75%, but of the additional 11 carcinogens, 10 had not been tested in the *in vitro* micronucleus test and so it is not known whether they would also have been positive in the *in vitro* micronucleus as well as in the MLA.

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

- Toluene was reported positive in the NTP MLA study, but has subsequently been re-evaluated as equivocal in the analysis of (Schisler et al., 2010), and was not found positive in a rigorous MLA conducted to higher concentrations and >80% toxicity (Kirkland and Fowler, 2010).
- Benzyl acetate was reported positive in the NTP MLA study, and subsequently re-evaluated as positive by Schisler et al (2010), but an expert panel review (Mitchell et al, 1997) identified this chemical as "untestable" in the MLA

because it reacts with the plastic of the culture vessels and may thus produce artefacts.

- Morphine was negative in a non-standard *in vitro* micronucleus test in which mouse splenocytes were treated only for 21 hr in the absence of metabolic activation. It is possible that morphine may induce MN when tested at higher concentrations over shorter periods in the absence and presence of metabolic activation in a standard assay.
- Thiabendazole is an aneugen which, typically, has a very steep dose response. It has been found positive for induction of MN *in vitro* in several papers, but is reported equivocal or negative in other papers, possibly because optimum concentration spacing, treatment and sampling times were not used.

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

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

**Annex 3 Table 1: Further data on combinations of genotoxicity tests**

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

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

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

112. Data presented to COM at the 17 June 2010 meeting (<http://webarchive.nationalarchives.gov.uk/20140506144349/http://www.iacom.org.uk/papers/documents/MUT2010-08Slides.pdf>) was published (Kirkland, 2011) and subsequently up-dated in light of re-evaluation of NTP MLA results (Schisler et al., 2010)

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### GLOSSARY

**Adduct:** A chemical grouping which is covalently bound to a large molecule such as DNA or protein.

**Alkylating agents:** Chemicals which leave an alkyl group covalently bound to biologically important molecules such as proteins and nucleic acids (see adduct). Many alkylating agents are mutagenic, carcinogenic and immunosuppressive.

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

**Aneugenic:** Inducing aneuploidy (qv).

**Aneuploidy:** The circumstances in which the total number of chromosomes within a cell is not an exact multiple of the normal haploid (see 'polyploidy') number. Chromosomes may be lost or gained during cell division.

**Apoptosis:** A form of active cell death resulting in fragmentation of the cell into membrane-bound fragments (apoptotic bodies). These are usually rapidly removed *in vivo* by engulfment by phagocytic cells. Apoptosis can occur normally during development, but is often triggered by toxic stimuli.

**Cancer:** Synonym for a malignant neoplasm – that is, a tumour (qv) that grows progressively, invades local tissues and spreads to distant sites (see also tumour).

**Carcinogenesis:** The origin, causation and development of tumours (qv). The term applies to benign as well as malignant neoplasms and not just to carcinomas

**Carcinogenicity bioassay:** Tests carried out in laboratory animals, usually rats and mice, to determine whether a chemical is carcinogenic. The test material is given throughout life to groups of animals at different dose levels.

**Carcinogens:** The causal agents which induce tumours. They include external factors (chemicals, physical agents, viruses) and internal factors such as hormones. Chemical carcinogens are structurally diverse and include naturally-occurring chemicals as well as synthetic compounds. An important distinction can be drawn between genotoxic (qv) carcinogens which have been shown to react with and mutate DNA, and non-genotoxic carcinogens which act through other mechanisms. The activity of genotoxic carcinogens can often be predicted from their chemical structure - either of the parent compound or of active metabolites (qv). Most chemical carcinogens exert their effects after prolonged exposure, show a dose-response relationship and tend to act on a limited range of susceptible target tissues. Carcinogens are sometimes species or sex-



specific and the term should be qualified by the appropriate descriptive adjectives to aid clarity. Several different chemical and other carcinogens may interact, and constitutional factors (genetic susceptibility, hormonal status) may also contribute, emphasising the multifactorial nature of the carcinogenic process.

**Chromosomal aberrations:** Collective term of particular types of chromosome damage induced after exposure to exogenous chemical or physical agents which damage the DNA. (see clastogen).

**Chromosome:** In simple prokaryotic organisms, such as bacteria and most viruses, the chromosome consists of a single circular molecule of DNA containing the entire genetic material of the cell. In eukaryotic cells, the chromosomes are thread-like structures, composed mainly of DNA and protein, which are present within the nuclei of every cell. They occur in pairs, the numbers varying from one to more than 100 per nucleus in different species. Normal somatic cells in humans have 23 pairs of chromosomes, each consisting of linear sequences of DNA which are known as genes.

**Clastogen:** An agent that produces chromosome breaks and other structural aberrations such as translocations. Clastogens may be viruses or physical agents as well as chemicals. Clastogenic events play an important part in the development of some tumours.

**Cytogenetic:** Concerning chromosomes, their origin, structure and function.

**Cytochrome P450 (CYP):** An extensive family of haem-containing proteins involved in enzymic oxidation of a wide range of endogenous and xenobiotic (qv) chemicals and their conversion to forms that may be more easily excreted. In some cases the metabolites produced may be reactive and may have increased toxicity. In other cases the chemicals may be natural precursors of hormones (e.g. steroids).

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

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

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

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

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

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

**Genome:** All the genetic material in the chromosomes of a particular organism; its size is generally given as its total number of base pairs.

**Genotoxic:** The ability of a chemical to cause DNA damage, either directly or after metabolic activation (see also carcinogens).

**Genotype:** The particular genetic pattern seen in the DNA of an individual. 'Genotype' is usually used to refer to the particular pair of alleles that an individual possesses at a certain location in the genome. Compare this with phenotype.

**Germ cell:** A biological cell that gives rise to the gametes of an organism that reproduces sexually. The cells undergo mitotic and meiotic cell division in the gonads followed by cellular differentiation into mature gametes, either oocytes or sperm.

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

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

***In vitro* chromosomal aberration assay:** An assay where cultured cell lines or human lymphocytes are incubated with test chemical. At a predetermined time, cells are arrested in metaphase, harvested and stained, and the metaphase spreads are then analysed microscopically for the presence of chromosome aberrations.

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

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

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

**Metabolic activation:** Metabolism of a compound leading to an increase in its activity, whether beneficial (e.g. activation of a pro-drug) or deleterious (e.g. activation to a toxic metabolite).

**Metabolic activation system:** A cell-free preparation (e.g. from the livers of rats pre-treated with an inducing agent added to *in vitro* tests to mimic the metabolic activation typical of mammals.

**Metabolism:** Chemical modification of a compound by enzymes within the body, for example by reactions such as hydroxylation (see cytochrome P450), epoxidation or

conjugation. Metabolism may result in activation, inactivation, accumulation or excretion of the compound.

**Metabolite:** Product formed by metabolism of a compound.

**Micronuclei:** Isolated or broken chromosome fragments which are not expelled when the nucleus is lost during cell division, but remain in the body of the cell forming MN. Centromere positive MN contain DNA and/or protein material derived from the centromere. The presence of centromere positive MN following exposure to chemicals *in vitro* or *in vivo* can be used to evaluate the aneugenic (qv) potential of chemicals.

**Micronucleus test:** See Micronuclei.

**Mitogen:** A stimulus which provokes cell division in somatic cells.

**Mitosis:** The type of cell division which occurs in somatic cells when they proliferate. Each daughter cell has the same complement of chromosomes as the parent cell.

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

**Mouse lymphoma assay:** An *in vitro* assay for gene mutation in mammalian cells using a mouse lymphoma cell line L5178Y, which is heterozygous for the gene (carries only one functional gene rather than a pair) for the enzyme thymidine kinase (TK<sup>+/-</sup>). Mutation of that single gene is measured by resistance to toxic trifluorothymidine. Mutant cells produce two forms of colony - large, which represent mutations within the gene and small, which represent large genetic changes in the chromosome such as chromosome aberrations. Thus this assay can provide additional information about the type of mutation which has occurred if colony size is scored.

**Mutation:** A permanent change in the amount or structure of the genetic material in an organism or cell, which can result in a change in phenotypic characteristics. The alteration may involve a single gene, a block of genes, or a whole chromosome. Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of large changes, including deletions, within the gene.

## DRAFT DOCUMENT FOR DISCUSSION

Changes involving whole chromosomes 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 end-points:** these comprise of three levels of genetic change, namely gene mutation, clastogenicity and aneuploidy

**No observable effect concentration (NOEC):** the highest administered concentration at which no adverse effect or specific genotoxic effect is seen

**Phenotype:** The observable physical, biochemical and physiological characteristics of a cell, tissue, organ or individual, as determined by its genotype and the environment in which it develops.

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

**32P-postlabelling:** A sensitive experimental method designed to measure low levels of DNA adducts induced by chemical treatment.

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

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

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

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

## DRAFT DOCUMENT FOR DISCUSSION

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

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

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

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

**Structure Activity Relationships:** the relationship between chemical structure and genotoxic effect based on predictions using computerised models (also Quantitative Structure Activity Relationships)

**Test chemical:** A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the chemical or changing its composition.

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

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

**Toxicogenomics:** A new scientific subdiscipline that combines the emerging technologies of genomics and bioinformatics to identify and characterise mechanisms of action of known and suspected toxicants. Currently, the premier toxicogenomic tools

are the DNA microarray and the DNA chip, which are used for the simultaneous monitoring of expression levels of hundreds to thousands of genes.

**Toxicokinetics:** The description of the fate of chemicals in the body, including a mathematical account of their absorption, distribution, metabolism and excretion. (see pharmacokinetics)

**Transgenic:** Genetically modified to contain genetic material from another species (see also genetically modified organism).

**Transgenic rodent gene mutation models:** Animals which have extra (exogenous) fragments of DNA incorporated into their genomes. This includes transgenic mice containing reporter genes to assess *in-vivo* mutagenicity in recoverable bacterial gene (*lacZ* or *lac I*). DNA can be isolated from a wide range of tissues following exposure to a test chemical and the genes assessed for induced mutations.

**Translation:** In molecular biology, the process during which the information in mRNA molecules is used to construct proteins.

**Tumour (Synonym - neoplasm):** A mass of abnormal, disorganised cells, arising from pre-existing tissue, which are characterised by excessive and uncoordinated proliferation and by abnormal differentiation. **Benign** tumours show a close morphological resemblance to their tissue of origin; grow in a slow expansile fashion; and form circumscribed and (usually) encapsulated masses. They may stop growing and they may regress. Benign tumours do not infiltrate through local tissues and they do not metastasise. They are rarely fatal. **Malignant tumours** (synonym - cancer) resemble their parent tissues less closely and are composed of increasingly abnormal cells in terms of their form and function. Well differentiated examples still retain recognisable features of their tissue of origin but these characteristics are progressively lost in moderately and poorly differentiated malignancies: undifferentiated or anaplastic tumours are composed of cells which resemble no known normal tissue. Most malignant tumours grow rapidly, spread progressively through adjacent tissues and metastasise to distant sites. Tumours are conventionally classified according to the anatomical site of the primary tumour and its microscopical appearance, rather than by cause.

**Unscheduled DNA Synthesis (UDS):** DNA synthesis that occurs at some stage in the cell cycle other than the S period (the normal or 'scheduled' DNA synthesis period), in response to DNA damage. It is usually associated with DNA repair.



## DRAFT DOCUMENT FOR DISCUSSION

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