MUT/2019/12

Deleted: 03

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

Commented [RB1]: Changes only up to para 75

Amend ref list – need endnote file

Check glossary and add links as needed

Also need to amend Figs 1 – 3 as needed

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

DRAFT DOCUMENT FOR DISCUSSION

GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALS

TABLE OF CONTENTS

Commented [RB2]: This version contains all changes agreed ...
Commented [RB3]: I have taken out use of substance through ...
Deleted: SUBSTANCES
Commented [RB4]: To be updated following agreement of th ...
Field Code Changed ...
Deleted: 3
Deleted: 5
Field Code Changed ...
Field Code Changed ...
Deleted: 6
Field Code Changed ...
Deleted: 8
Field Code Changed ...
Deleted: 8
Deleted: 10
Field Code Changed ...
Field Code Changed ...
Deleted: 11
Field Code Changed ...
Deleted: 11
Field Code Changed ...
Deleted: 12
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...
Deleted: 21
Field Code Changed ...
Deleted: *21*
Deleted: *21*
Field Code Changed ...
Field Code Changed ...
Deleted: 24
Field Code Changed ...
Deleted: *24*
Field Code Changed ...
Deleted: *25*
Field Code Changed ...
Deleted: *25*
Field Code Changed ...
Deleted: *25*
Field Code Changed ...
Deleted: *26*
Field Code Changed ...
Deleted: 27
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...
Deleted: 32
Field Code Changed ...
Deleted: 33
Field Code Changed ...
Deleted: *33*
Field Code Changed ...
Deleted: 33
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...
Field Code Changed ...

Field Code Changed

Deleted: 46

Deleted: 49

Field Code Changed

Field Code Changed

Deleted: 58

DRAFT DOCUMENT FOR DISCUSSION

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[1]. 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

[1] Note that the terms 'test' and 'assay' are used interchangeably throughout the document to reflect naming conventions.

Deleted: SUBSTANCES

Deleted: 2000

Deleted: ,

Deleted: ,

Deleted:

Commented [RB5]: Link to document when online

Commented [RB6]: Link to document when online

Commented [RB7]: Link to doc when available

Commented [RB8]: Link to doc when available

Formatted: Highlight

Deleted: testing of nanomaterials is not covered in this guidance. Developments in the use of Quantitative Structure Activity Relationship (QSAR) modelling and the applicability of *in vivo* assays were considered in 2018.

Commented [RB9]: Ref neeed

Deleted: T

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.

Commented [RB10]: Can this be defined??

Commented [RB11]: Genotoxicity?

Commented [RB13]: This requires updating once the amendments to the document are agreed.

Deleted: <#>It is acknowledged that the field of genotoxicology and genotoxicity testing is rapidly developing. Some possible future developments and techniques such as toxicogenomics are discussed. ¶
<#>

DRAFT DOCUMENT FOR DISCUSSION

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). 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.

Deleted: Chief Medical Officer for England

Deleted:

Deleted: C

Deleted: all

Deleted: 8-9

Deleted: Directorate

Commented [RB14]: Should this just be genotoxicity??

Commented [RB15]: Link to Glossary added

Deleted: substances

Field Code Changed

Deleted: substance

Deleted: . In this document the term test substance

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

Deleted: However, t

Deleted: substance

Commented [RB16]: Also remove link

Deleted: or pure substance

Field Code Changed

Commented [RB17]: Delete this as not longer needed?

Commented [RB18]: Amend ref when changes complete

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).

Commented [RB19]: Amend ref when changes complete

Commented [RB20]: Amend ref when changes complete

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.

Deleted: The rationale developed by COM in 2000, particularly in relation to the testing of all potential mutagenic endpoints, has also been was adopted by the International Workshops on Genotoxicity Testing (IWGT) (Muller et al., 2003) and the strategy developed by COM in 2011 has been adopted by EFSA and REACH (ECHA, 2017; EFSA, 2011).

Deleted:

Deleted: have no place in the recommended

Deleted: testing strategy

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.

Deleted: substance

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.

Deleted: substances

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.

Deleted: substance

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).

Field Code Changed

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.

Deleted: substances

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[2].

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

Deleted: and
Deleted: effects
Deleted: mutagenicity
Deleted: *in vivo* genotoxicity testin
Deleted: substances
Deleted: substances
Field Code Changed
Field Code Changed

[2] 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

Field Code Changed

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; Matthews et al., 2006). However, experience suggests that mammalian cell tests conducted and interpreted according to current recommendations perform more robustly (Clarke et al., 2012).

Deleted: substances

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).

Deleted: substance

Commented [RB21]: Sarah – is this a good place to mention this and link to the separate doc?
Also - can you confirm the title

Commented [RB22]: Add link when available

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

Deleted: substance

Deleted: substances

Deleted: substance

(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

Deleted: substances

Commented [RB23]: Some text has been removed so that this is more of a short summary with ref to stand alone doc

Suggested deletions – to be agreed

Deleted: substance

Commented [RB24]: Link to document when online

Commented [RB25]: Is the title correct?

Deleted: A composite model structure was originally devised by Ashby and Tennant indicating substituent chemical groups or moieties associated with DNA-reactivity (Ashby and Paton, 1993). A number of freely available and commercial systems to investigate SAR for mutagenicity have been developed and evaluated since 2000 (Benigni and Bossa, 2008; Benigni et al., 2007; Cariello et al., 2002; Contrera et al., 2005; Snyder and Smith, 2005; Zeiger et al., 1996). Further information on various models is provided in Annex 1. The OECD (OECD, 2004) and the European Commission (Joint Research Centre (JRC)) have published principles for the validation of QSAR (Benigni et al., 2010; Worth et al., 2005) (!!! INVALID CITATION !!! (Worth et al., 2005, Benigni and Bossa, 2008))¶
. QSAR assessment of the in *vitro* mutagenicity in bacteria has been attained by two types of approach; statistical analyses of structure and mutagenic activity and/or QSAR models using programmed rules for prediction of mutagenic activity based on the available knowledge and expert judgement. ¶
Such QSAR systems can be useful when a large number of chemicals require assessment and prioritisation for genotoxicity testing or in instances where a rapid assessment of a chemical is required and there are no genotoxicity test data available. Each QSAR system has a defined domain of applicability which is determined by the structural/descriptor factors, modes/mechanism of mutagenicity, and metabolic aspects included within the system. In addition *in silico* approaches can aid in the interpretation of Stage 1 *in vitro* genotoxicity test results (Dearfield et al., 2010). The available systems perform well for prediction of bacterial mutagenicity (i.e. for chemical structures within the domain of applicability of the model under consideration) (see Annex 1). However, lower sensitivities and specificities have been reported for a number of systems when used for prediction of results from *in vitro* cytogenetics or the mouse lymphoma assay (e.g. using MCASE and MDL-QSAR) (Contrera et al., 2008a). One factor in the lower predictive capability of QSAR systems for mammalian cell genotoxicity assays is inadequate coverage of non-covalent DNA interactions and non-DNA targets associated with cell division (Grant et al., 2000; Snyder and Smith, 2005). It has also been proposed that QSAR assessments can aid in the interpretation of the relevance of *in vitro* genotoxicity assays through prediction of biotransformation (Combes et al., 2007). Other systems combining metabolic simulation with structure toxicity rules have been developed (e.g. TIMES; tissue metabolic simulator) but are at a relatively early stage of validation (Mekenyan et al., 2004; Serafimova et al., 2007). Lhasa Ltd has developed a computer programme (METEOR), which has the facility to integrate prediction of metabolism with QSAR approaches for genotoxicity. (https://www.lhasalimited.org/products/meteor-nexus.htm). An authoritative and comprehensive evaluation of the different QSAR approaches to the identification of …

Commented [RB26]: Link to document when online

Deleted: although

Deleted: However, expert judgement is needed when reaching conclusions on mutagenic hazard on the basis of QSAR information alone

Commented [RB27]: It was suggested to have this as a separate document – do we want to do this – not sure what the decision was??

I can revisit section once confirmed

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).

Deleted: substances

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).

Field Code Changed

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 *Bscl2* 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).

Field Code Changed

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.

Deleted: substance

Formatted: Font: Italic

Commented [RB28]: Add Matthews et al., 2006

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;

Commented [RB29]: Add to refs

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).

Commented [RB30]: Add to refs

Commented [RB31]: Add link when available.

Deleted: such as

Deleted: for which OECD guidelines have been deleted.

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.

Formatted: Highlight

Deleted: substances

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).

Formatted: Highlight

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.

Deleted: substances

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)

Commented [RB32]: Do members have any other/updated references?

Commented [DK33]: There are probably many we could add. A PubMed search of "indirect genotoxicity" brought up 238 references! t would take time decide which are most relevant.

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).

Deleted:

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

Deleted: will be due

Deleted: to

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$_5$) (Emmert et al., 2006), the use of exogenous metabolic activation systems derived from human sources, or recombinant human cytochrome P450 systems as an external activation system (Ku et al., 2007b).

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

Deleted: personal communication to author

Commented [RB34]: Insert from endnote when finalised

Deleted: this change to

Deleted: in OECD guidelines

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).

Commented [RB35]: Amend to 2012b

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).

Commented [RB36]: Amend to 2012a

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:

- 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.

Deleted: substance

Commented [RB37]: Question from DK: Should we add in the latest OECD recommendations for obtaining and monitoring historical control data?

Deleted: substance

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 (WP2*uvrA* or WP2*uvrA* (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).

Commented [RB38]: This section needs updating but need information from an IGWT doc to be published in 2-3 months.

Deleted: In order t

Deleted: substance

Commented [DK39]: Should we also mention Ames MPF since it includes all of the regulatory strains whereas AmesII does not?

Deleted:)

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; Pfuhler 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

Commented [RB40]: Sarah – is this discussed in the nano doc??

Deleted: although it is emphasised that such materials are not within the scope of this guidance.

Commented [RB41]: Is background information prior to 2011 still needed?

Commented [DK42]: I would say not needed.

Commented [RB43]: Which to delete?

Deleted: .¶

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 ± 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 pan-centromeric 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

Deleted:

Deleted: Careful selection of toxicity measurements has been shown to reduce the potential for misleading positive results (Fowler et al., 2010b).

Deleted: can be combined

Deleted:

Commented [RB44]:

Commented [DK45]: We might want to separate the use of pan-centromeric or anti-kinetochore antibodies to determine chromosome loss into MN from use of whole chromosome probes to determine non-disjunction in daughter nuclei. At the moment it is a bit muddled.

Commented [RB46]: Text amended

Deleted: centromere or kinetochore stains, with pancentromeric or chromosome specific centromeric probes using fluorescence *in situ* hybridisation (FISH) as a sensitive way to discriminate between chromosome breaks, chromosome loss and chromosome non-disjunction and polyploidy (Kirsch-Volders et al., 2002) and therefore is useful in assessing mode of action (Parry, 2006).

Deleted: and

Commented [RB47]: Does this paragraph reflect the many updates for flow assays that have happened since this draft?

Commented [DK48]: Probably not. A PubMed search of "flow cytometry micronuclei in vitro" brought up 90 references!

Commented [RB49]: Can members suggest key papers to cite?

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.

Commented [RB50]: Should this example be removed as now many other examples available?

65. An interlaboratory evaluation of the MultiFlowVR DNA Damage kit— p53, gH2AX, 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, gH2AX, 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

Deleted: The available data indicate that the *in vitro* metaphase analysis and the *in vitro* micronucleus assay have similar overall performance for determination of clastogenicity.

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.

Commented [RB51]: Update ref to 2018
Evaluation of U. S. National Toxicology Program (NTP) mouse lymphoma assay data using International Workshop on Genotoxicity Tests (IWGT) and the Organization for Economic Co-Operation and Development (OECD) criteria.
Schisler MR, Gollapudi BB, Moore MM.
Environ Mol Mutagen. 2018 Dec;59(9):829-841.

Deleted: 2010

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).

Deleted: sensitivity

Field Code Changed

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.

Deleted: and the assay is reported to result in fewer misleading positive results due to cytotoxicity or precipitation than CA tests

Deleted: substances

Commented [DK52]: Should we add the latest IWGT report from the 2017 meeting?

Commented [RB53]: Can you provide the ref please?

Commented [RB54]: Still awaiting publication

Deleted: substances

Deleted:

Commented [RB55]: Comment from GC: Not sure that this follows, given the discussion on the "oversensitivity" of batteries of mammalian tests, cytotoxicity issues and the fate of the lesions.

Commented [RB56]: How to address this??

Deleted: However, since the comet assay does not detect aneuploidy, and may report repairable DNA damage, it is not recommended as a core *in vitro* test.

Summary Stage 1 (*In Vitro* Genotoxicity Testing)

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.

Deleted: Information

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

Commented [RB57]: Following sections were not reviewed in the meeting

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

Commented [RB58]: Link to standalone germ cell doc??

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

Deleted: mutagenic

Commented [DK60]: Should we say "genotoxic potential"?

Commented [RB61]: amended

Deleted: mutagenicity

Deleted: points 1)-5)

Deleted: substance

Deleted: 2000

Formatted: Font: Italic

Formatted: Font: Italic

Commented [DK63]: I presume we need to talk about changes since 2011 and not since 2000?

Commented [RB64]: amended

Commented [RB65]: check number

Deleted: tissue

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

Deleted: substance

Deleted: EFSA,

Commented [RB66]: Should 'Factorial Design' be included here?

Deleted:

Commented [RB67]: Comment from GC: add "to integrate"

Deleted: combining micronucleus and cComet assays into a single acute test employing repeat a few administrations of test chemical

Deleted: C

Commented [K68]: Question forMembers: Can this be updated to include comet?

Commented [RB69]: Committee answer - no

Deleted: slides

Deleted: scoring

Deleted: substances

Commented [RB70]: Question from DK: Do we want to say anything about i.p. versus physiologically relevant?

Formatted: Highlight

Deleted: 62

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

Commented [RB71]: Question from DK: Or toxicity data in the target tissue from other studies? Use the recommendations in Hardy et al, 2017?

Deleted: substances

Commented [RB72]: Question from DK: Do we want to add that based on GTTC and IWGT analyses, comet is as sensitive as transgenic for the key tissues of liver and GI tract, and for detecting bacterial mutagens, and mutagenic carcinogens? However, these analyses don't tell us anything about specificity.

Deleted:

Deleted: mutagenic

Deleted: mutagenic

Deleted: substances

Commented [DK73]: Wouldn't aneuploidy in germ cells also be a hazard? However, nut "mutagenic".

Deleted: substances

Deleted: substances

Deleted: substances

Commented [BS74]: formatting

Deleted: #81

Deleted: (Tweats et al., 2007b)

Deleted: substance

Deleted: (Muller et al., 2003)

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).

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

Commented [RB75]: Question from DK: Do we bring in reference to the quantitative dose response analysis approaches, determination of PoD etc?

Field Code Changed

Commented [RB76]: Linked checked and OK

Deleted: (Blakey et al., 2008; Tweats et al., 2007a; Shuey et al., 2007)

Deleted: genotoxic mode(s) of action

Field Code Changed

Commented [RB77]: Link checked and OK

Formatted: Highlight

Deleted: 66

Deleted: substance

Commented [RB78]: Add link when available.

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

Deleted: C

Commented [DK79]: Presumably this refers to the transgenic assay – we can probably update this now.

Commented [RB80]: Link to standalone germ cell doc needed

Deleted: although

Deleted: in vivo

Deleted: Attia et al., 2005)

Deleted:

Formatted: Highlight

Commented [RB82]: Keep text here

Deleted: A summary of this evaluation and the Committees conclusions on testing is in preparation

Deleted: : OR>

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.

Commented [RB83]: Comment from GC: but the currently accepted tests for assessing germ cell mutagenicity are underutilised and there are few investigations examining the possibility

Commented [RB84]: Incomplete sentence?

Deleted: The COM considers that further research to understand better the effects of genotoxic substances on mammalian germinal cells may be informative with regard to genotoxicity testing strategies in the future. It is noted that some initial results with oocyte *in vitro* and *in vivo* systems have provided information on germ-cell specific modes of action (Ranaldi et al., 2008; Vogt et al., 2008; Yin et al., 1998).

Deleted: recent

Deleted:

Deleted: micronuclei

Deleted:

Commented [RB85]: Check correct once editing complete

Deleted: 61-73)

Deleted:

Deleted:

Deleted: complimentarity

Commented [DK86]: We probably need to include reference to Morita et al 2016 here as well.

Commented [RB87]: added

Commented [RB88]: To include GTTC and IWGT findings comparing TGR and comet if agree to add earlier in text.

Commented [DK89]: This whole section may need significant revision.

Commented [RB90]: Standalone doc for germ cell mutagens has been prepared – need to decide how to link from main document

Deleted: substance

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 in 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

Commented [DK91]: I think we need to reduce the emphasis on bone marrow since blood MN tests are now very common. Suggest MNviv to cover both.

Commented [RB92]: amended

Deleted: BM

Deleted:

Deleted: BM

Deleted:

Moved (insertion) [5]

Deleted:

Field Code Changed

Deleted: BM

Deleted: micronuclei (

Deleted:)

Deleted: (

Deleted:)

Deleted:

Deleted: numerical aberrations

Deleted: Micronuclei

Commented [DK93]: In flow cytometry of blood it is DNA content. This needs to be updated.

Commented [RB94]: Suggested text needed??

Deleted: BM

Moved up [5]: 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).

Deleted: mutagenic

Commented [RB95]: DK Comment: there are more recent publications.

RB: Suzuki et al 2008 Mutagenesis 24?
Please provide others

Commented [DK96]: I am not sure we want to mention liver here – maybe later. There are several publications on the liver MN test that can be cited.

Commented [RB97]: Can members provide key refs??

Deleted: young

Deleted: (Suzuki et al., 2005)

Deleted:

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™mouse, BigBlue® mouse and rat (including use of λ 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

Deleted: (Singer et al., 2006)

Commented [DK98]: As earlier, I think we can update this.

Commented [RB99]: See previous

Deleted: I

Deleted: n addition t

Deleted: can be

Commented [DK100]: Isn't this superceded now by the OECD guideline?

Commented [RB101]: Members??

Deleted: Salmonella

Commented [DK102]: May need to update with latest IWGT analysis.

Commented [RB103]: See previous comment re standalone doc

Commented [DK104]: Again, this can probably be updated.

Commented [RB105]: See previous

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).

Deleted: <#>http://www.oecd-ilibrary.org/environment/test-no-488-transgenic-rodent-somatic-and-germ-cell-gene-mutation-assays_9789264122819-en ¶

Deleted: C

Deleted: C

Deleted: substances

Formatted: Highlight

Commented [RB106]: David – can you provide refs for these

Commented [DK107]: Yes, once they are finalised – hopefully not too long.

Commented [RB108]: amended

Deleted: substance

Deleted: There is now consensus agreement on a protocol for most tissues which would be consistent with an OECD guideline (Burlinson et al., 2007).

Commented [RB109]: DK comment: IWGT analysis indicates liver comet combined with bone marrow or blood MN will be sufficient in most cases. If systemic exposure is expected or found to be low, then site-of-contact effects in stomach or duodenum (no evidence that both are needed) are effective.

RB: text change needed??

Deleted: C

Commented [RB110]: DK Comment: Can refer to GTTC and IWGT reports.

RB: can you provide refs to these

Commented [DK111]: As above, once finalised.

Deleted: With regard to the assessment of germ cell genotoxicity, measurement of DNA effects by the Comet assay in sperm requires additional steps for chromatin decondensation.

Deleted: A protocol for standardisation of the germ cell Comet assay has not yet been achieved (Speit et al., 2009).

Deleted: can be used

Deleted: and i

Deleted: C

Deleted: within standard regulatory toxicity tests (Rothfuss et al., 2010) or

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*.

Deleted: gives broadly similar results to the

Deleted: EFSA

Deleted: 's preference is to

Deleted: *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

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:

Commented [RB112]: Link to specific documents

Table 1 Supplementary in vivo genotoxicity tests

Commented [DK113]: I have not reviewed this yet.

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA Adducts				
^{32}P-postlabelling	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 ^{32}P. (Phillips et al., 2000)
Covalent binding to DNA A variety of methods can be used such as those involving radioactive decay measurements (eg. ^{14}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)
Supplementary investigations of germ cell mutagenicity				
Analysis for clastogenicity/ aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes	OECD	Can provide information on nature of effects in spermatogonia, spermatocytes and/or oocytes of mice or rats	Can provide useful information on MoGA. (Russo, 2000)
Spermatid micronucleus assay	Chromosomal aberrations and or lagging chromosomes	None available	Provides information of clastogenic and/or aneugenic effects in spermatocytes.	(Allen et al., 2000)
Dominant lethal assay	Chromosomal/gene mutations	OECD	Provides information on unstable chromosomal changes in gametes that lead to fetal death after fertilization and can determine stage(s) of gametogenesis affected	Little used. needs relatively large numbers of animals (Adler et al., 1994)
Mouse specific locus test	Gene mutations	EPA	Provides information on genetic changes transmitted to the first generation progeny as basis for estimation of induced mutation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on chromosomal changes transmitted to the first generation progeny as basis for estimation of induced translocation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Sperm Comet assay	Double strand breaks and/or apurinic sites in sperm head DNA	None available	Provides information on genetic instability in sperm	(Trivedi et al., 2010)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes	EPA	Provides information on induction of DNA lesions	(Sotomajor and Sega, 2000)

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

Deleted: mutagenic

Commented [DK115]: As earlier – suggest "genotoxic potential"

Commented [RB116]: amended

Deleted: mutagenicity

Deleted: mutagenic

Deleted: substance

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

Commented [K117]: Question for COM: In view of OECD GL development should Pig-A be considered as a supplementary test rather than in future developments section?

Commented [DK118]: Yes.

Commented [RB119]: Comment from DK: yes it should be a supplementary test.

RB: Move section??

Deleted:

Commented [RB120]: To David – can you provide a ref please

Deleted: or

Deleted: and was considered by COM in 2014.

Deleted: (COM minutes 2014) https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/690196/COM_meeting_minutes_March_2014.pdf

Deleted: (Singer et al., 2006)

Commented [RB121]: Comment from GC: is it worth summarising why not?

Deleted: (COM papers MUT/08/14 and MUT/09/03) (http://webarchive.nationalarchives.gov.uk/20140506144419/http://www.iacom.org.uk/papers/documents/ToxicogenomicsMUT.08.14.pdf; http://webarchive.nationalarchives.gov.uk/20140506144500/http://www.iacom.org.uk/papers/documents/toxicogenom2009MUT09.03.pdf).

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).

Commented [RB122]: Comment from GC: mention epigenetics, if only to say that beyond the scope of this guidance?

Annex 1. Sensitivity and Specificity Data Considered by the COM

Commented [RB123]: No updates to be done past this point until revisions completed for remaining document.

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.

Field Code Changed

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

Commented [K124]: To be updated in line with para 30-34 when amended

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

Annex 1 Table 2 Screening Tests

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

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
In vitro assays		
Ames	Gene mutation	Responds to wide range of DNA reactive mutagens when full set of *S. typhimurium* tester strains and *E. coli* 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.
In vivo 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.

Field Code Changed

Deleted: MUT/2010/08).

Deleted: Kirkland et al paper

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.

Deleted: micronuclei

Deleted: micronuclei

Deleted: MNvit

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

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

Deleted:

Field Code Changed

References

Aardema, M., Albertini, S., Arni, P., Henderson, L., Kirsch-Volders, M., Mackay, J., Sarrif, D., Stringer, D., and Taalman, R. (1998). Aneuploidy: a report of an ECETOC task force Mutation Research *410*, 3-79.

Aardema, M., Snyder, R., Spicer, C., Divi, K., Morita, T., Mauthe, R., Gibson, D., Soelter, S., Curry, P., Thybaud, V., *et al.* (2006). SFTG international collaborative study on *in vitro* micronucleus test. III. Using CHO cells. Mutation Research *607*, 61-87.

Aardema, M.J., Barnett, B.B., Mun, G.C., Dahl, E.L., Curren, R.D., Hewitt, N.J. and Pfuhler, S. (2013). Evaluation of chemicals requiring metabolic activation in the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay. Mutation Research *750*, 40-49.

Ackerman, J., Sharma, R., Hitchcock, J., Hayashi, T., Nagai, Y., Li, S., Lu, S., Miret, J., Tang, K., Spence, F., *et al.* (2009.). Inter-laboratory evaluation of the bioluminescent Salmonella reverse mutation assay using 10 model chemicals. Mutagenesis *24*, 433-438.

Adler, I.-D. (2008). Mutagenicity Tests *in vivo*. In Toxicology and Risk Assessment H. Grein, Snyder, R. , ed. (Wiley and Sons, UK), pp. pp371-384.

Adler, I., Shelby, M., Bootman, J., Favor, J., Generoso, W., Pacchierotti, F., Shibuya, T., and Tanaka, N. (1994). Summary report of the Working Group on Mammalian Germ Cell Tests. Mutation Research *312*, 313-318.

Allen, J.W., Collins, B.W., Lan, A., Afshari, A.J., George, M.H., DeAngelo, A.B., and Fuscoe, J.C. (2000). Erythrocyte and spermatid micronucleus analyses in mice chronically exposed to potassium bromate in drinking water. Environmental and Molecular Mutagenesis *36*, 250-252.

Ashby, E., and Paton, D. (1993). The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 differnet human carcinogen exposures Mutation Research *286*, 3-74.

Attia, S., Badary, O., Hamada, S., de Angelis, M., and Adler, I. (2005). Othovanadate increased the frequency of aneupolid mouse sperm without micronucleus induction in mouse bone marrow erythroctes at the same dose level. Mutation Research *583*, 158-167.

Aubrecht, J., Osowski, J., Persaud, P., Cheung, J., Ackerman, J., Lopes, S., and Ku, W. (2007). Bioluminescent Salmonella reverse mutation assay: a screen for detecting mutagenicity with high throughput attributes. Mutagenesis *22*, 335-342.

Benfenati, E., Benigni, R., Demarini, D., Helma, C., Kirkland, D., Martin, T., Mazzatorta, P., Ouédraogo-Arras, G., Richard, A., Schilter, B., *et al.* (2009). Predictive models for carcinogenicity and mutagenicity: frameworks, state-of-the-art, and perspectives. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. *27*, 57-90.

Benigni, R., and Bossa, C. (2008). Structure alerts for carcinogenicity, and the Salmonella assay system: a novel insight through the chemical relational databases technology. Mutation Research *659*, 248-261.

Benigni, R., Bossa, C., and Worth, A. (2010). Structural ananlysis and predictive vlaue of the rodent *in vivo* micronucleus assay. Mutagenesis *e-pub March 1*, 1-7.

Benigni, R., Netzva, T., Benfenati, E., Bossa, C., Franke, R., Helma, C., Hulzebos, E., Marchant, C., Richard, A., Woo, Y.-T., *et al.* (2007). The Expanding Role of Predictive Toxicology: An Update on the (Q)SAR Models for Mutagens and Carcinogens. Journal of Environmental Science, Health C Environ Carcinog Ecotoxicol Rev. *25*, 53-97.

Commented [RB125]: Still to be formatted and refs in red need adding to EndNote file - to be done once all changes are complete

All refs need checking against main text – to be done once all changes are complete

Commented [DK126]: Need to add ICH M7 and M7(R1) refs.

Commented [RB127]: Added

Deleted: !!! INVALID CITATION !!! (Garriott et al., 2002, Lorge et al., 2006, Oliver et al., 2006, Wakata et al., 2006)).¶
(!!! INVALID CITATION !!! (Worth et al., 2005, Benigni and Bossa, 2008)).

Birrell, L., Cahill, P., Hughes, C., Tate, M., and Walmsley, R. (2010). AM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. Mutation Research *695*, 87-95.

Bishop, J. (2003). Female specific reproductive toxicities following preconception exposuer to xenobiotics. Advances in Experimental Medicine *518*, 1-9.

Blakey, D., Galloway, S., Kirkland, D., and MacGregor, J. (2008). Regulatory asepcts of genotoxicity testing: from hazard identification to risk assessment. Mutation Research *657*, 84-90.

Bowen, D., Whitwell, J., Lillford, L., Henderson, D., Kidd, D., Mc Garry, S., Pearce, G., Beevers, C., and Kirkland, D. (2010). Validation of a multi-endpoint assay in rats: Bone marrwo micronculeus, comet and flow cytometric peripheral blood micronucleus. Mutation Research (accepted for publication).

Bowen, D., Whitwell, JH, Lillford, L, Henderson, D, Kidd, D, Mc Garry, S, Pearce, G, , and Beevers, C., Kirkland, DJ; (2011). Evaluation of a multi-endpoint assay in rats, combining the bone-marrow

micronucleus test, the Comet assay and the flow-cytometric peripheral blood

micronucleus test. Mutation Research

xx, xx.

Brambilla, G., and Martelli, A. (2004). Failure of the standard battery of short-term tests in detecting some rodent and human genotoxic carcinogens. Toxicology *196*, 1-19.

Bryce, S., Avlasevich, S., Bemis, J., Lukamowicz, M., Elhajouji, A., Van Goethem, F., De Boeck, M., Beerens, D., Aerts, H., van Gompel, J., *et al.* (2008a). Interlaboratory evaluation of a flow cytometric, high content *in vitro* micronucleus assay. Mutation Research *650*, 181-195.

Bryce, S., Bemis, J., Avlasevich, S., and Dertinger, S. (2007). *In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutation Research *78-91*.

Bryce, S., Benis, J., and Dertinger, S. (2008b). *In vivo* Mutation Assay based on the Endogenous *Pig-a* Locus. Environmental and Molecular Mutagenesis *49*, 256-264.

Bryce, S., Shi, J., Nicolette, J., Diehl, M., Sonders, P., Avlasevich, S., Raja, S., Bemis, J., and Dertinger, S. (2010). High Content Flow Cytometric Micronucleus Scoring Method is Applicable to Attachment Cell Lines.

. Environmental and Molecular Mutagenesis *51*, 260-266.

Burlinson, B., Tice, R., Speit, G., Agurell, E., Brendler-Schwaab, S., Collins, A., Escobar, P., Honma, M., Kumaravel, T., Nakajima, M., *et al.* (2007). Fourth International Workgroup on Genotoxicity testing: Results of the *in vivo* Comet assay workgroup. Mutation Research *627*, 31-35.

Cariello, N., Wilson, J., Britt, B., Wedd, D., Burlinson, B., and Gombar, V. (2002). Comparison of the computer programs DEREK and TOPKAT to predict bacterial mutagenicity. Mutagenesis *17*, 321-329.

Chapman, K, Thomas A, Wills, J, Pfuhler, S, Doak, S, Jenkins G. (2014) Automation and validation of micronucleus detection in the 3D EpiDerm™ human reconstructed skin assay and correlation with 2D dose responses. Mutagenesis *29*, 165-175.

Cimino, M. (2006). Comparative Overview of Current International Strategies and Guidelines for Genetic Toxicology Testing for Regulatory Purposes. Environmental and Molecular Mutagenesis *47*, 362-390.

Ciranni, R., Antonetti, M., and Miglore, L. (1995). Vanadium salts induce cytogenetic effects in *in vivo* treated mice. Mutation Research *343*, 53-60.

Clare, M., Lorenzon, G., Akhurst, L., Marzin, D., van Delft, J., Montero, R., Botta, A., Bertens, A., Cinelli, S., Thybaud, V., *et al.* (2006). SFTG international collaborative study on *in vitro* micronucleus test II. Using human lymphocytes. Mutation Research *607*, 37-60.

Clarke, J, Lawlor, T, Madraymootoo, W, Pant, K, Young, R, Wagner, V, 3rd, Aardema, M. (2012). Summary of in vitro genetic toxicology assay results: Expected and unexpected effects of recent study design modifications. Environ Mol Mutagen *53*, 631–635.

Claxton, L., Stewart-Hook, V., and Warren, S. (2001). Methods for the Spiral *Salmonella* mutagenicity assay including specilaised applications. . Mutation Research *488*, 241-257.

Clements, J. (2000). The Mouse Lymphoma Assay. Mutation Research *455*, 97-110.

Combes, R., Grindon, C., Cronin, M., Roberts, D., and Garrod, J. (2007). Proposed Integrated Decision-tree Testing Strategies for Mutagenicity and Carcinogenicity in Relation to the EU REACH Legislation. . ATLA *35*, 267-287.

Contrera, J., Mathews, E., Kruhlak, N., and Benz, R. (2005). *In silico* screening of Chemicals for Bacterial Mutagenicity Using Electropological E-state indices and MDL QSAR Software. Regulatory Toxicology Pharmacology *43*, 313-323.

Contrera, J., Matthews, E., Kruhlak, N., and Benz, R. (2008). *In silico* Screening of Chemicals for Genetic Toxicity Using MDL-QSA, Nonparametric Discriminant Analysis, E-State, Connectivity and Molecular Property Descriptors. Toxicology Mechanisms and Methods *18*, 207-216.

Corvi, R., Albertini, S., Hartung, T., Hoffman, S., Muaurici, D., Pfuhler, S., van Benthem, J., and Vanparys, P. (2008). ECVAM retrospective validation of the *in vitro* micronucleus test (MNT). Mutagenesis *23*, 271-283.

CSGMT (1995). The Collaborative Study Group for the Micronucleus Test (CSGMT)[1] (CSGMT/JEMS.MMS, The Mammalian Study Group of the Environmental Mutagen Society of Japan). Protocol recommended by the CSGMT/JEMS.MMS for the short-term mouse peripheral blood micronucleus test. Mutagenesis *10*, 153-159.

Curren, R., Mun, G., Gibson, D., and Aardema, M. (2006). Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm™). Mutation Research *607*, 192-204.

Custer, L., and Sweder, K. (2008). The role of Genetic Toxicology in Drug Discovery and Optimization. Current Drug Metabolism. *9*, 978-985.

De Boeck, M., van der Leede, B., Van Goethem, F., De Smedt, A., Steemans, M., A., L., and Vanparys, P. (2005). Flow cytometric analysis of micronucleated reticulocytes: Time- and dose-dependent response of known mutagens in mice, using multiple blood sampling. Environmental Molecular Mutagenesis *46*, 30-42.

Dean, S., Brooks, T., Burlinson, B., Mirsalis, J., Myhr, B., Recio, L., and Thybaud, V. (1999). Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing for *in vivo* for the detection of site-of-contact mutagens. Mutagenesis *14*, 141-151.

Dearfield, K., Thybaud, V., Cimino, M., Custer, L., Czich, A., Harvey, J., Hester, S., Kim, J., Kirkland, D., Levy, D., *et al.* (2010). Follow-up Actions from Positive Results of *In Vitro* Genetic Toxicology Testing.

Dearfield, K.L., Gollapudi, B.B., Bemis, J.C., Benz, R.D., Douglas, G.R., Elespuru, R.K., Johnson, G.E., Kirkland, D.J., LeBaron, M.J., Li, A.P., *et al.* (2017). Next generation testing strategy for assessment of genomic damage: A conceptual framework and considerations. Environ Mol Mutagen *58*, 264-283.

Decordier, I., Papine, A., Plas, G., Roesems, S., Vande Loock, K., Moreno-Palomo, J., Cemeli, E., Anderson, D., Fucic, A., Marcos, R., *et al.* (2009). Automated image analysis of cytokinesis-blocked micronuclei: an adapted protocol and a validated scoring procedure for biomonitoring. . Mutagenesis *24*, 85-93.

Dertinger, S., Phonethepswath, S., Franklin, D., Weller, P., Torous, D., Bryce, S., Avlasevich, S., Bemis, J., Hyrien, O., Palis, J., *et al.* (2010). Integration of mutation and chromosomal damage endpoints into 28-day repeat dose toxicology studies. Toxicological Sciences *115*, 401-411.

Dertinger, S.D., Bryce, S.M., Phonethepswath, S., and Avlasevich, S.L. (2011). When pigs fly: immunomagnetic separation facilitates rapid determination of *Pig-a* mutant frequncy by flow cytometric analysis. . Mutation Research. *721*, 163-170.

DOH (1989). Department of Health. Report on Health and Social Subjects, 35. Guidelines for the testing of chemicals for Mutagenicity. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. London, HMSO.

DOH (2000). Department of Health. Guidance for the testing of chemicals for Mutagenicity. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment.

DOH (2011). Department of Health. Guidance on a strategy for genotoxicity testing of chemicals. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. Eastmond, D., Hartwig, A., Anderson, D., Anwar, W., Cimino, M., Dobrev, I., Douglas, G., Nohmi, T., Phillips, D., and Vickers, C. (2009). Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonised Scheme. Mutagenesis *24*, 341-349.

ECHA (2017). Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance, version 6. July 2017 R.7.7 Available from : https://www.echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf/e4a2a18f-a2bd-4a04-ac6d-0ea425b2567f

EFSA (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 9, 2379. https://www.efsa.europa.eu/en/efsajournal/pub/2379.

EFSA Scientific Committee, Hardy A, Benford D, Halldorsson T, Jeger M, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Younes M, Aquilina G, Crebelli R, Gurtler R, Hirsch-Ernst KI, Mosesso P, Nielsen E, van Benthem J, Carf M, Georgiadis N, Maurici D, Parra Morte J and Schlatter J, (2017). Scientific Opinion on the clarification of some aspects related to genotoxicity assessment. EFSA Journal 2017;15(12):5113, 25 pp. Available: https://www.efsa.europa.eu/en/efsajournal/pub/5113.

Eichenlaub-Ritter, U., Adler, I., Carere, A., and Pacchierotti, F. (2007). Gender differences in germ cell mutagenesis and genetic risk. Environmental Research *104*, 22-36.

Elespuru, R.A., R. Atrakchi, AH. Bigger, CA. Heflich, RH. Jagannath, DR. Levy,, and DD. Moore, M.O., Y, Robison, TW, Sotomayor, RE, Cimino, MC, Dearfield KL. (2009). Current and future application of genetic toxicity assays: the role and value of

in vitro mammalian assays. Toxicological Sciences *109*, 172-179.

Emmert, B., Bunger, J., Keuch, K., Muller, M., Emmert, S., Hallier, E., and Westphal, G. (2006). Mutagenicity of cytochrome P450 2E1 substrates in the Ames test with metabolic compentent *S. typhimurium* strain YG7108pin3ERb$_5$. Toxicology *228*, 66-76.

Escobar, P.A., Kemper, R.A., Tarca, J., Nicolette, J., Kenyon, M., Glowienke, S., Sawant, S.G., Christensen, J., Johnson, T.E., McKnight, C., Ward, G., Galloway, S.M., Custer, L., Gocke, E., O'Donovan, M.R., Braun, K., Snyder, R.D., Mahadevan, B. (2013). Bacterial mutagenicity screening in the pharmaceutical industry. Mutation Research/Reviews in Mutation Research *752*, 99-118.

Deleted: ¶

Deleted: ¶

Field Code Changed

Field Code Changed

Deleted: EFSA (2017). Clarification of some aspects related to genotoxicity assessment':

Deleted: available

Field Code Changed

Fellows, M., O'Donovan, M., Lorge, E., and Kirkland, D. (2008a). Comparison of different methods for an accurate assessment of cytotoxicity in the in vitro micronucleus test II. Practical aspects with toxic agents. Mutation Research *655*, 4-21.

Fellows, M., O'Donovan, M., Lorge, E., and Kirkland, D. (2008b). Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus testII: Practical aspects with toxic agents. Mutation Research *655*, 4-21.

Fellows, M.D., Boyer, S., and O'Donovan, M.R. (2011a). The incidence of positive results in the mouse lymphoma TK assay (MLA) in pharmaceutical screening and their prediction by MultiCase MC4PC. . Mutagenesis *26*, 529-532.

Fellows, M.D., Doherty, A.T., Priestley, C.C., Howarth, V. and O'Donovan, M.R. (2011b). The ability of the mouse lymphoma TK assay to detect aneugens. Mutagenesis *26*, 771-781.

Fenech, M. (2006). Cytokinesis-block micronucleus assay evolves into a 'cytome' assay for chromsomal instability, mititoc dysfunction and cell death. Mutation Research *600*, 58-66.

Fenech, M. (2007). Cytokinesis-block micronucleus assay. Nature Protocols *2*, 1084-1104.

Fischer, S.J., Benson, L.M., Fauq, A., Naylor, S., and Windebank, A.J. (2008). Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. . NeuroToxicology *29*, 444-452.

Flamand, N., Marrot, L., Belaidi, J.-P., Bourouf, L., Dourille, E., Feltes, M., and Meunier, J.-R. (2006). Development of genotoxicitiy test procedures with Episkin® a reconstructed human skin model: Towards new tools for *in vitro* risk assessment of dermally applied compounds? Mutation Research *606*, 39-51.

Fluckigetr-Isler, S., Baumeister, M., Braun, K., Gervais, V., Hasler-Nguyen, N., Reimann, R., Van Gompel, J., Wunderlich, H.-G., and Engelhardt, G. (2004). Assessment of the performance of the Ames II^{TM}assay: a collaborative study with 19 coded compounds. Mutation Research *558*, 181-197.

Fowler, P., Young, J., Jeffrey, L., Hand, T., Smith, K., Kirkland, D., Carmichael, P., and Pfuhler, S. (2012a). Reduction of misleading("false") positive results in mammalian cell genotoxicity assays I. Choice of cell type. Mutat Res. *742*, 11-25..

Fowler, P., Young, J., Jeffrey, L., Hand, T., Smith, K., Kirkland, D., Carmichael, P., and Pfuhler, S. (2012b). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays II. The importance of cytotoxicity measure. Mutat Res. *747*, 104-17.Galloway, S. (2000). Cytotoxicity and Chromosome Aberrations *In Vitro*: Experience in Industry andthe Case for an Upper Limit on Toxicity in the Aberration Assay. Environmental and Molecular Mutagenesis *35*, 191-201.

Galloway, S., Lorge, E, Aardema, MJ, Eastmond, D, Fellows, M, Heflich, R, Kirkland, D,, and Levy, D., Lynch, AM, Marzin, D, Morita, T, Schuler, M, Speit, G. (2011). Workshop summary: Top concentration for *in vitro* mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for *in vitro*

cytogenetics assays (chromosome aberrations and micronucleus). Mutation Research *xx*, xx.

Garriott, M., Phelps, J., and Hoffman, W. (2002). A protocol for the in vitro micronucleus test I. Contributions to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanisms of action and different levels of activity. Mutation Research *517*, 123-134.

Gatehouse, D., Haworth, S., Cebula, T., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Ohta, T., and Venitt, S. (1994). Recommendations for the performance of bacterial mutation assays. Mutation Research *312*, 217-233.

Deleted: Fowler, P. (2009). Industrial Genotoxicity Group, November 2009. Reduction of False/Misleading Positives in in-vitro genetic toxicology testing: Importance of cell selection and toxicity measure. .¶
Fowler, P., Wiiliams, K., Jeffrey , L., Carmichael, P., Ardema, M., Diembeck, W., Fautz, R., Harvey, J., Hewitt, N., Latil, A., *et al.* (2009a). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays I. Choice of cell type. Environmental Molecular Mutagenesis *50*, 572.¶
Fowler, P., Wiiliams, K., Jeffrey , L., Carmichael, P., Ardema, M., Diembeck, W., Fautz, R., Harvey, J., Hewitt, N., Latil, A., *et al.* (2009b). Reduction of misleading("false") positive results in mammalian cell genotoxicity assays II. Importance of aaccurate toxicity measurement. . Environmental Molecular Mutagenesis *50*, 573.¶

Deleted: 2010a

Deleted: a

Deleted: In preparation.¶

Deleted: Fowler, P., Young, J., Jeffrey, L., Hand, T., Smith, K., Kirkland, D., Carmichael, P., and Pfuhler, S. (2010b2012b). Reduction of misleading("false") positive results in mammalian cell genotoxicity assays I. Choice of cell type. Mutat Res. *742*, 11-25.In preperation.¶

Gealy, R., Wright-Bourque, J., Kraynak, A., McKelvey, T., Barnum, J., and Storer, R. (2007). Validation of a high-throughput *in vitro* alkaline elution/rat hepatocyte assay for DNA damage. Mutation Research *629*, 49-63.

Glei, M., Hovhannisyan, G., and Pool-Zobel, B. (2009). Use of Comet-FISH in the study of DNA damage and repair: Review. Mutation Research *681*, 33-43.

Grant, S., Zhang, Y., Klopman, G., and Rosenkranz, H. (2000). Modeling the mouse lymphoma forward mutational assay: the Gene-Tox program database. . Mutation Research *465*, 201-229.

Hamada, S., Sutou, S., Morita, T., Wakata, A., Asanami, S., Hosoya, S., Ozawa, S., Kondo, K., Nakajima, M., Shimada, H., *et al.* (2001). Evaluation of the Rodent Micronucleus Assay by a 28-Day Treatment Protocol: Summary of the 13th Collaborative Study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)-Mammalian Mutagenicity Study Group (MMS). Environmental and Molecular Mutagenesis *37*, 93-110.

Hartmann, A., Kiskinis, E., Fjallman, A., and Suter, W. (2001). Influence of cytotoxicity and compound precipitation on test results in the alkaline comet assay. . Mutation Research *497*, 199-212.

Hartmann, A., Schumacher, M., Plappert-Helberg, U., Lowe, P., Suter, W., and Mueller, L. (2004). Use of the alkaline *in vivo* Comet assay for mechanistic genotoxicity investigations. Mutagenesis *19*, 51-59.

Hastwell, P., Chai, L., Roberts, K., Webster, T., Harvey, J., Rees, R., and Walmsley, R. (2006). High-specificity and high sensitivity genotoxicicty assessment in a huma cell line: Validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. Mutation Research *607*, 160-175.

Hayashi, M., Dearfield, K., Kasper, P., Lovell, D., Martus, H.J., and Thybaud, V. (2011). Compilation and use of genetic toxicity historical control data. Mutat Res *723*, 87-90.

Hayes, J.E., Doherty, A.T., Coulson, M., Foster, J.R., Cotton, P.T., and O'Donovan, M.R. (2013). Micronucleus induction in the bone marrow of rats by pharmacological mechanisms. I: glucocorticoid receptor antagonism. Mutagenesis *28*, 227-232.

Helleday, T., Johansson, F., and Jenssen, D. (2001). The DRAG Test: an Assay for Detection of genotoxic Damage. ATLA *29*, 233-241.

Hendriks, G., Atallah, M., Morolli, B., Calleja, F., Ras-Verloop, N., Huijskens, I., Raamsman, M., van de Water, B., and Vrieling, H. (2012). The ToxTracker assay: novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicol Sci *125*, 285-298.

Hendriks, G., Atallah, M., Raamsman, M., Morolli, B., van der Putten, H., Jaadar, H., Tijdens, I., Esveldt-van Lange, R., Mullenders, L., van de Water, B., *et al.* (2011). Sensitive DsRed fluorescence-based reporter cell systems for genotoxicity and oxidative stress assessment. Mutat Res *709-710*, 49-59.

Himmelstein, M., Boogaard, P., Cadet, J., Farmer, P., Kim, J., Martin, E., Persaud, R., and Shuker, D. (2009). Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. . Critical Reviews in Toxicology. *39*, 679-694.

Hiramoto, K., Nasuhara, A., Michikoshi, K., Kato, T., and Kikugawa, K. (1997). DNA strand-breaking activity and mutagenicity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4-H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. . Mutation Research *395* 47-56.

Hu, T., Kaluzhny, Y., Mun, G., Barnett, B., Karetsky, V., Wilt, N., Klausner, M., Curren, R., and Aardema, M. (2009). Intralaboratory and interlaboratory evlauation of the EpiDerm™ 3D human reconstructed skin micronucleus assay. Mutation Research *673*, 100-108.

ICH (2011). Guidance on Genotoxiicty Testing and Data Interpretation for Pharmaceuticals Intended for Human Use . S2(R1) Available: http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html

Field Code Changed

ICH (2012). ICH guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use. Available: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/12/WC500119604.pdf.

ICH (2014). Harmonised tripartite guidline on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential cancer risk - M7. Available: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M7/M7_Step_4.pdf [accessed Jan 2019].

ICH (2015). Harmonised guideline - addendum to ICH M7: assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential cancer risk. Application of the principles of the ICH M7 guideline to calculation of compound-specific acceptable intakes - M7(R1). Available: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M7/M7_Addendum_Step_2.pdf [accessed Jan 2019].

Jagger, C., Tate, M., Cahill, P., Hughes, C., Knight, A.W., Billinton, N., and Walmsley, R.M. (2008). Assessment of the genotoxicity of S9-generated metabolites using the GreenScreen HC GADD45a-GFP assay. Mutagenesis *24*, 35-50.

Jarabek, A., Pottenger, L., Andrews, L., Casciano, D., Embry, M., Kim, J., Preston, J., Vijayaraj Reddy, M., Schoeny, R., Shuker, D., *et al.* (2009). Creating context for use of DNA adduct data in cancer risk assessment: I Data organization. Critical Reviews in Toxicology *39*, 659-678.

Kamber, M., Fluckiger-Isler, S., Engelhardt, G., Jaeckh, R., and WZeiger, E. (2009). Comparison of the Ames II and the traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. . Mutagenesis *24*, 359-366.

Kasper, P., Uno, Y., Mauthe, R., Asano, N., Douglas, G., Matthews, E., Moore, M., Mueller, L., Nakajima, M., Singer, T., *et al.* (2007). Follow-up testing of rodent carcinogens not positive in the standard genotoxicity test battery: IWGT workgroup report. Mutation Research *627*, 106-116.

Kennelly, J., Water, R., Ashby, J., Lefevre, P., Burlinson, B., Benford, D., Dean, S., and Mitchell I, d. (1993). *In vivo* rat liver UDS assay. In Supplementary Mutagenicity Tests. UKEMS Report. Cambridge University Press pp 52-77.

Khanal, S., Singh, P., Avlasevich, S.L., Torous, D.K., Bemis, J.C., and Dertinger, S.D. (2018). Integration of liver and blood micronucleus and Pig-a gene mutation endpoints into rat 28-day repeat-treatment studies: Proof-of-principle with diethylnitrosamine. Mutat Res *828*, 30-35.

Kirkland, D. (2010). Evaluation of different cytotoxic and cytostatic measures for the *in vitro* micronucleus test (MNVit). Summary of results of collaborative trial. Mutation Research *702*, 139-147.

Kirkland, D., Aardema, M., Henderson, L., and Muller, L. (2005a). Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens 1. Sensitivity, specificity and relative predictivity. Mutation Research *584*, 1-256.

Kirkland, D., Henderson, L., Marzin, D., Muller, L., Parry, J., Speit, G., Tweats, D., and Williams, G. (2005b). Testing strategies in mutagenicity and genetic toxicology: An appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutation Research *588*, 88-105.

Kirkland, D., Aardema, M., Banduhn, N., Carmichael, P., Fautz, R., Meunier, J., and Pfuhler, S. (2007a). *In vitro* approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive *in vitro* genotoxicity results. Mutagenesis *22*, 161-175.

Moved (insertion) [1]

Deleted: Kirkland, D. (2010). Evaluation of different cytotoxic and cytostatic measures for the *in vitro* micronucleus test (MNVit). Summary of results of collaborative trial. . Mutation Research *in press doi:10.1016/j.mrgentox.2010.02.001*.¶

Moved up [1]: Kirkland, D., Aardema, M., Henderson, L., and Muller, L. (2005a). Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens 1. Sensitivity, specificity and relative predictivity. Mutation Research *584*, 1-256.¶

Moved (insertion) [2]

Moved down [3]: Kirkland, D., and Fowler, P. (2010). Further analysis of Ames-negative rodent carcinogens that are only genotoxic in mammalian cells at concnetrations exceeding 1mM including retest of compounds of concern. In preparation.¶

Kirkland, D., Hayashi, M., Jacobson-Kram, D., Kasper, P., MacGregor, J., Muller, L., and Uno, Y. (2007b). Summary of major conclusions from the 4th IWGT, San Francisco, 9-10 September, 2005. Mutation Research *627*, 5-9.

Kirkland, D., Pfuhler, S., Tweats, D., Aardema, M., Corvi, R., Darroudi, F., Elhajouji, A., Glatt, H., Hastwell, P., Hayashi, M., *et al.* (2007c). How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid uncessessary follow-up animal tests: Report of an ECVAM Workshop. Mutation Research *628*, 31-55.

Kirkland, D., and Speit, G. (2008). Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutation Research *654*, 114-132.

Kirkland, D., and Fowler, P. (2010). Further analysis of Ames-negative rodent carcinogens that are only genotoxic in mammalian cells at concnetrations exceeding 1mM including retest of compounds of concern. Mutagenesis *25*, 539-553.

Kirkland, D., Reeve, L., Gatehouse, D., and Vanparys, P. (2011). A core *in vitro* genotoxicity battery comprising the Ames test plus the *in vitro* micronucleus test is sufficient to detect rodent carcinogens and *in vivo* genotoxins. Mutation Research *721*, 27-73.

Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M.J., Kirchner, S., Lorge, E., Morita, T., *et al.* (2003). Report from the in vitro micronucleus assay workshop group. Mutation Research *540*, 153-163.

Kirsch-Volders, M., Vanhauwaert, A., DeBoeck, M., and Decordier, I. (2002). Importance of detecting numerical versus structural chromsome aberrations. Mutation Research *504*, 137-148.

Kirsch-Volders, M., Vanhauwaert, A, Eichenlaub-Ritter, U, Decordier, I. (2003). Indirect mechanisms of genotoxicity. Toxicology Letters *140-141*, 63-74.

Knight, A., Little, S., Houck, K., Dix, D., Judson, R., Richard, A., McCarroll, N., Akerman, G., Yang, C., Birrell, L., *et al.* (2009). Evaluation of high-throughpuyt genotoxicity assays used in profiling the US EPA ToxCast^{TM} chemicals. Regulatory Toxicology Pharmacology *55*, 188-199.

Krishna, G., Urda, G., and Theiss, J. (1998). Principles and Practices of Integrating Genotoxicity Evaluation Into Routine Toxicology Studies: A Pharmaceutical Industry perspective. Environmental and Molecular Mutagenesis *32*, 115-120.

Ku, W., Aubrecht, J., Mauthe, R., Schiestl, R., and Fornace Jr, A. (2007a). Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part VII: Why Not Start with a Single Test: A Transformational Alternative to Genotoxicity Hazard and Risk Assessment. Toxicological Sciences *99*, 20-25.

Ku, W., Bigger, A., Brambilla, G., Glatt, H., Gocke, E., Guzzie, P., Hakura, A., Honma, M., Martus, H., Scott Obach, R., *et al.* (2007b). Strategy for genotoxicity testing-Metabolic considerations. Mutation Research *627*, 59-77.

Laingam, S., Froscio, S., and Humpage, A. (2008). Flow-cytometric analysis of *in vitro* micronucleus formation: Comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cells. Mutation Research *656*, 19-26.

Lambert, I., Singer, T., Boucher, S., and Douglas, G. (2005). Detailed review of transgenic rodent mutation assays. Mutation Research *590*, 1-280.

Moved up [2]: Kirkland, D., Henderson, L., Marzin, D., Muller, L., Parry, J., Speit, G., Tweats, D., and Williams, G. (2005b). Testing strategies in mutagenicity and genetic toxicology: An appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutation Research *588*, 88-105.¶

Deleted: Kirkland, D., Hayashi, M., Jacobson-Kram, D., Kasper, P., MacGregor, J., Muller, L., and Uno, Y. (2007c). Summary of major conclusions from the 4th IWGT, San Francisco, 9-10, 2005. Mutation Research *627*, 5-9.¶

Deleted: d

Moved (insertion) [4]

Moved (insertion) [3]

Deleted: In preparation.

Moved up [4]: Kirkland, D., and Speit, G. (2008). Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutation Research *654*, 114-132.¶

Deleted: Kirkland, D., Reeve, L., Gatehouse, D., and Vanparys, P. (2010). A core *in vitro* genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and *in vivo* genotoxins. Mutation Research (accepted for publication). .¶

Deleted: .

Le Hegarat, L.D., J; Josse, R; Huet, S; Lanceleur, R; Mourot, A; Poul, J; Guguen-Guillouzo, C; Guillouzo, A; Fessard, V (2010). Assessment of the genotoxic potential of indirect chemical mutagens in HepaRG cells by the comet and the cytokinesis-block micronucleus assays Mutagenesis *25*, 555-560.

Li, A.G., RS. Heflich, RH. Wassom JS. (1988). A review and analysis of the Chinese hamster ovary/hypoxanthine guanine phosphoribosyl transferase assay to determine the mutagenicity of chemical agents. A report of phase III of the U.S.

Environmental Protection Agency Gene-Tox Program. Mut.Res. *196*, 17-36.

Long, L., Kirkland, D., Whitwell, J., and Halliwell, B. (2007). Different cytotoxic and clastogenic effects of epigallocatechin gallate in various cell-culture media due to variable rates of oxidation in culture media. Mutation Research *634*, 177-183.

Lorge, E., Hayashi, M., Albertini, S., and Kirkland, D. (2008). Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. Mutation Research *655*, 1-3.

Lorge, E., Thybaud, V., Aardema, M., Oliver, J., Wakata, A., Lorenzon, G., and Marzin, D. (2006). SFTG international collaborative study on the *in vitro* micronucleus test I. General conditions and overall conclusions of the study. Mutation Research *607*, 13-36.

Lynch, A.M., Sasaki, J.C., Elespuru, R., Jacobson-Kram, D., Thybaud, V., De Boeck, M., Aardema, M.J., Aubrecht, J., Benz, R.D., Dertinger, S.D., *et al.* (2011). New and emerging technologies for genetic toxicity testing. Environmental and Molecular Mutagenesis *52*, 205-223.

Madrigal-Bujaidar, E., Madrigal-Santillan, E., Alvarez-Gonzalez, I., Baez, R., and Marquez, P. (2008). Micronuclei Induced by Imipramine and Desipramine in Mice: A Subcronic Study. Basic & Clinical Pharmacology & Toxicology. *103*, 569-573.

Maierhofer, C., Jentsch, I., lederer, G.F., C., and Speicher, M. (2002). Multicolor FISH in two and three diemnsions for clastogenic analysis. Mutagenesis *17*, 523-527.

Mathews, E., Kruhlak, N., Cimino, M., Benz, R., and Contrera, J. (2006). An analysis of genetic toxicology, reproductive and developmental toxicity and carcinogenicity data I: Idenitfication of carcinogens using surrogate end points. Regulatory Toxicology and Pharmacology *44*, 83-96.

Matthews, E., Kruhlak, N., Cimino, M., Benz, R., and Contrera, J. (2006). An analysis of genetic toxicology, reproductive and developmental toxicity and carcinogenicity data: II Identification of genotoxicants, reprotoxicants, and carcinogens using in silico methods. Regulatory Toxicology and Pharmacology *44*, 97-110.

Mekenyan, O., Dimitrov, S., Serafimova, R., Thompson, E., Kotov, S., Dimitrova, N., and Walker, J. (2004). Identification of the structural requirements for mutagenicity by incorporating molecular flexibility and metabolic activation of chemicals I: TA100 model Chemical Research and Toxicology *17*, 753-766.

Miller, J., Vlasakova, K., Glaab, W., and Skopek, T. (2005). A low volume, high-throughput forward mutation assay in Salmonella typhimurium based on fluorouracil resistance. Mutation Research *578*, 210-224.

Mitchell, I., Lambert, T., Burden, M., Sunderland, J., Porter, R., and Carlton, J. (1995). Is Polyploidy an important genotoxic lesion? Mutagenesis *10*, 79-83.

Miura, D., Dobrovolsky, V., Kimoto, T., Kasahara, Y., and Heflich, R. (2009). Accumulation and persistence of *Pig-A* mutant peripheral red blood cells following treatment of rats with a single and split doses of *N*-ethyl-*N*-nitrosourea. . Mutation Research *677*, 86-92.

Moore, M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J., Clay, P., Doppalapudi, R., Fellows, M., *et al.* (2007). Mouse Lymphoma thymidine kinase gene mutation assay:

Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. Mutation Research *627*, 36-40.

Moore, M., Honma, M., Clements, J., Bolcsfoldi, G., Cifone, M., Delongchamp, R., Fellows, M., Gollapudi, B., Jenkinson, P., Kirby, P., *et al.* (2003). Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: International Workshop on Genotoxicity Tests Workgroup Report-Plymouth, UK 2002. Mutation Research *540*, 127-140.

Moore, M., Honma, M, Clements, J, Bolcsfoldi, G, Burlinson B, Cifone M, Clarke J, , Delongchamp R, D.R., Fellows M, Gollapudi B, Hou S, Jenkinson P, Lloyd M,, Majeska J, M.B., O'Donovan M, Omori T, Riach C, San R, Stankowski LF Jr, Thakur, AK, V.G.F., Wakuri S, Yoshimura I. (2006). Mouse lymphoma thymidine kinase gene

mutation assay: follow-up meeting of the International Workshop on Genotoxicity

Testing--Aberdeen, Scotland, 2003--Assay acceptance criteria, positive controls,

and data evaluation. Environ.Mol.Mutagen. *47*, 1-5.

Morita, T., Miyajima, A., Hatano, A. and Honma, M. (2014). Effects of lowering the proposed top-concentration limit in an in vitro chromosomal aberration test on assay sensitivity and on the reduction of the number of false positives. Mutation Research *769*,34-49.

Morita, T., Hamada, S., Masumura, K., Wakata, A., Maniwa, J., Takasawa, H., Yasunaga, K., Hashizume, T., and Honma, M. (2016). Evaluation of the sensitivity and specificity of in vivo erythrocyte micronucleus and transgenic rodent gene mutation tests to detect rodent carcinogens. Mutat Res Genet Toxicol Environ Mutagen *802*, 1-29.

Muller, L., Blakely, D., Dearfield, K., Galloway, S., Guzzie, P., Hayashi, M., Kasper, P., Kirkland, D., MacGregor, J., Parry, J., *et al.* (2003). Strategy for genotoxicity and stratification of genotoxicity test results- report of initial activities of the IWGT Expert Group. Mutation Research *540*, 177-181.

Müller, L.K., P. (2000). Human biological relevance and the use of

threshold-arguments in regulatory genotoxicity assessment: experience with

pharmaceuticals. Mutation Research *464*, 19-34.

Mun, G., Aardema, M., Hu, T., Barnett, B., Kaluzhny, Y., Klausner, M., Karetsky, V., Dahl, E., and Curren, R. (2009). Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay. Mutation Research *673*, 92-99.

OECD (1997). Organisation for Economic Co-operation and Development. Ninth addendum to the OECD guidelines for the testing of chemicals. Update of Section 4, Health Effects. Revised guidelines 424, 471, 473, 474, 475, 476, 483, 486, 487

OECD (2004). OECD Series on testing and Assessment. Number 49. The report from the Expert Group on (Quantitative) Structure-Activity Relatiopnships [(Q)SARs] on the principles for the validation of (Q)SARs. (2nd Meeting of the Ad hoc Expert group on QSARs, OECD Headquaters, 20-21 September 2004. JT00176183, ENV/JM/MONO(2004)24, 17-Dec-2005.

OECD (2009). Detailed Review Paper On Transgenic Rodent Mutation Assays, Series On Testing And

Transgenic Rodent Gene Mutation Assay Dratft OECD Test Guideline Sept 15 version.doc

10 Assessment Number 103, OECD, Paris, July 23, 2009. http://www.olis.oecd.org/olis/2009doc.nsf/linkto/ENV-JM-MONO(2009)7.

Deleted: and

Field Code Changed

OECD (2013). OECD GUIDELINES FOR THE TESTING OF CHEMICALS Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays. Available: https://www.oecd-ilibrary.org/docserver/9789264203907-en.pdf?expires=1525085655&id=id&accname=guest&checksum=3A754D79F5D56195846182380E76D7DB.

OECD (2016a). OECD GUIDELINE FOR THE TESTING OF CHEMICALS In Vitro Mammalian Cell Micronucelus Test. Available: https://www.oecd-ilibrary.org/docserver/9789264264861-en.pdf?expires=1533801766&id=id&accname=guest&checksum=7E11CD6A97E99C8EE995DC7E46536D76.

OECD (2016b). OECD GUIDELINE FOR THE TESTING OF CHEMICALS In Vivo Mammalian Alkaline Comet Assay. Available: https://www.oecd-ilibrary.org/docserver/9789264264885-en.pdf?expires=1525089115&id=id&accname=guest&checksum=75B861F770C09914E3F613E68652F975.OECD (2016c), Test No. 473: In Vitro Mammalian Chromosomal Aberration Test, OECD Publishing, Paris. Available: https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosomal-aberration-test_9789264264649-en.

OECD (2016d) Test No. 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, OECD Publishing, Paris. Available: https://www.oecd-ilibrary.org/environment/test-no-490-in-vitro-mammalian-cell-gene-mutation-tests-using-the-thymidine-kinase-gene_9789264264908-en.

OECD (2016e), Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes, OECD Publishing, Paris. Available: https://www.oecd-ilibrary.org/environment/test-no-476-in-vitro-mammalian-cell-gene-mutation-tests-using-the-hprt-and-xprt-genes_9789264264809-en.

Ogawa, I., Furukawa, S., Abe, M., Tanaka, Y., and Hayashi, S.U., K. (2009). Multi-endpoint genotoxic assay usin L5178Y ($Tk^{+/-}$-3.7.2c) cells. The Journal of Toxicological Sciences *34*, 547-553.

Olaharski, A., Albertini, S., Kirchner, S., Platz, S., Uppal, H., Lin, H., and Kolaja, K. (2009). Evaluation of the GreenScreen GADD45a-GFP inidcator assay with non-proprietary and proprietary compounds. Mutation Research *672*, 10-16.

Oliver, J., Meunier, J.-R., Awogi, T., Elhajouji, A., Ouldelhkim, M.-C., Bichet, N., Thybaud, V., Lorenzon, G., Marzin, D., and Lorge, E. (2006). SFTG international collaborative study on in vitro micronucleus test V. Using L5178Y cells. Mutation Research *607*, 125-152.

Pacchierotti, F., Adler, I.-D., Eichenlaub-Ritter, U., and Mailher, J.B. (2007). Gender effects on the incidence of aneuploidy in mammalian germ cells. Environ.Res. *104*, 46-69.

Parry, J. (2006). The use of the *in vitro* micronucleus assay to detect and assess the aneugenic activity of chemicals. Mutation Research *607*, 5-8.

Parry, J., Parry, E., Phrakonkham, P., and Corvi, R. (2010). Analysis of published data for top concnetrations in mammalian cell genotoxicity testing. Mutagenesis *25*, 531-538.

Pfuhler, S. (2009). 5th International Workshop on Genotoxicity testing, August 17-19, 2009. Topic 3: In vitro test approaches with better predictivity. ICEM, Florence, August 20-25, 2009.

Pfuhler, S., Albertini, S., Fautz, R., Herbold, B., Madle, S., Utesch, D., and Poth, A. (2007). Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evlauation Part IV: Recomendation of a Working Group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a Simple and Straightforward Approach to Genotoxicity Testing. Toxicological Sciences *97*, 237-240.

Pfuhler, S., Fellows, M., van Benthem, J., Corvi, R., Curren, R., Dearfield, K., Fowler, P., Frotschl, R., Elhajouji, A., Le Hegarat, L., *et al.* (2011). *In vitro* genotoxicity test approaches with better predictivity: Summary of an IWGT workshop. Mutatation Research *723*, 101-107.

Field Code Changed

Field Code Changed

Deleted: ¶

Pfuhler, S., Kirkland, D., Hayashi, M., Vanparys, P., Carmichael, P., Dertinger, S., Eastmond, D., Elhajouji, A., Krul, C., Rothfuss, A., *et al.* (2009). Reduction of use of animals in regulatory genotoxicity testing: Identification and implementation opportunities- Report from an ECVAM workshop. Mutation Research *680*, 31-42.

Pfuhler, S., Kirst, A., Aardema, M., Banduhn, N., Garsten, G., Daisuke, A., Costabel-Farkas, M., Dufour, E., Fautz, R., Harvey, J., *et al.* (2010). A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: Genotoxicity. A COLIPA analysis.

Phelps, J., Garriott, M., and Hoffman, W. (2002). A protocol for the in vitro micronucleus test II. Contributions to the validation of a protocol suitable for regulatory submissions from an examination of 10 chemicals with different mechansims of action and different levels of activity. Mutation Research *521*, 103-112.

Phillips, D., Farmer, P., Beland, F., Nath, R., Poirier, M., Reddy, M., and Turteltaub, K. (2000). Methods of DNA adduct determination and their application to testing compounds for genotoxicity. Environ Mol Mutagen *35*, 222-233.

Pratt, I.B., T. (2003). Regulatory recognition of indirect genotoxicity mechanisms in the European Union. Toxicology Letters *140-141*, 53-62.

Ponton, I., Mutch, P., Nicholls, D.J., Saad, A., Diaz Pohl, C., Young, A., Fred, C., O'Donovan, M.R., and Aberg, P. (2013). Micronucleus induction in the bone marrow of rats by pharmacological mechanisms. II: long-acting beta-2 agonism. Mutagenesis *28*, 233-239.

Ranaldi, R., Gambuti, G., Eichenlaub-Ritter, U., and Pacchierotti, F. (2008). Triclorfon effects on mouse oocytes following in vivo exposure Mutation Research *651*, 125-130.

Rapp, A., Bock, C., Dittmar, H., and Greulich, K.-O. (2000). UV-A breakage sensitivty of human chromosomes as measured by COMET-FISH depends on gene density and not on chromosome size.

. Journal of Photochemistry and Photobiology B: Biology *56*, 109-117.

Reifferscheid, G., Arndt, C., and Schmid, C. (2005). Further Developmen of the β-lactamase Mutagen Assay and Evlauation by Comparison with Ames Fluctuation Tests and the umu Test. Environmental and Molecular Mutagenesis *46*, 126-139.

Reisinger, K, Blatz, V, Brinkmann, J, Downs, T, Fischer, A, Henkler, F, Hoffmann, S, Krul, C, Liebsch, M, Luch, A, Pirow, R, Reus, A, Schulz, M, Pfuhler, S. (2018) Validation of the 3D Skin Comet assay using full thickness skin models: Transferability and reproducibility. Mutation Research/Genetic Toxicology and Environmental Mutagenesis *827*, 27-41.

Roithfuss, A., Steger-Hartmann, T., Heinrich, N., and Wichard, J. (2006). Conspirational Prediction of the Chromosome-Damaging Potential of Chemicals. Chem Res Toxicol *19*, 1313-1319.

Rothfuss, A., Honma, M., Czich, A., Aardema, M., Burlinson, B., Galloway, S., Hamada, S., Kirkland, D., Heflich, R., Howe, J., *et al.* (2010). Improvement Of *In Vitro* Genotoxicity Assessment: Combination of Acute Tests and the Link to Standard Toxicity Testing. Mutation Research (accepted for publication).

Rothfuss, A., Honma, M, Czich, A, Aardema, MJ, Burlinson, B, Galloway, S, Hamada, S, Kirkland, D, Heflich, RH, Howe, J, Nakajima, M, O'Donovan, M, Plappert-Helbig, U, Priestley, C, Recio, L, Schuler, M, Uno Y, Martus, HJ (2011). Improvement of *in vivo* genotoxicity assessment: Combination of acute tests and integration into standard toxicity testing. Mutation Research *xx*, xx.

Roy, S., Kulkarni, R., Hewitt, N.J. and Aardema, M.J. (2016). The EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay: Historical control data and proof of principle studies for mechanistic assay adaptations. Mutation Research *805*, 25-37.Russo, A. (2000). In vivo cytogenetics in mammalian germ cells. Mutation Research *455*, 167-189.

Formatted: Font: Italic

Deleted: ¶

Santos, S.J., Singh, N.P., and Natarajan, A.T. (1997). Flourescence in situ hybridization with comets. . Experimental Cell Research *232*, 407-411.

SCCNFP (2003). The Scientific Committee on Cosmetic Products and Non-food Products intended for Consumers. Updated recommended strategy for testing hair dyes for their potential genotoxicity/mutagenicity/carcinogenicity. Available: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out224_en.pdf

SCCS (2016). The Scientific Committee on Consumer Safety. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation. Available: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf [accessed Aug 2018].

Schisler, M., Gollupudi, B., and Moore, M. (2010). Evaluation of the Mouse Lymphoma Mutation Assay (MLA) Data of the U.S. National Toxicology Program (NTP) Using International Workshop on Genotoxicity Tests (IWGT) Criteria. Environmental Molecular Mutagenesis *51*, P96.

Serafimova, R., Todorov, M., Pavlov, T., Kotov, S., Jacob, E., Aptula, A., and Mekenyan, O. (2007). Identification of the structural requirements for mutagenicity by incorporating molecular flexibility and metabolic activation of chemicals. II. General Ames and mutagenicity model Chemical Research and Toxicology *20*, 662-676.

Shuey, D.L., Gudi, R., Krsmanovic, L., and Gerson, R.J. (2007). Evidence that oxymorphone-induced increases in micronuclei occur secondary to hyperthermia. Toxicol Sci *95*, 369-375.

Singer, T., Lambert, I., Williams, A., Douglas, G., and Yauk, C. (2006). Detection of induced male germline mutation: Correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. Mutation Research *598*, 164-193.

Snyder, R., and Smith, A. (2005). Computational prediction of genotoxicity: room for improvement. Drug Discovery Today (Biosilico) *10*, 1119-1124.

Sofuni, T., Honma, M, Hayashi, M, Shimada, H, Tanaka, N, Wakuri, S, Awogi, T, Yamamoto,KI, Nishi, Y, Nakadate, M. (1996). Detection of in vitro clastogens and spindle poisons by

the mouse lymphoma assay using the microwell method: interim report of an

international collaborative study. Mutagenesis *11*, 349-355.

Sotomajor, R.E., and Sega, G.A. (2000). Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update. Environ.Mol.Mutagen. *36*, 255-265.

Spanswick, V., Hartley, J., and Hartley, J. (2010). Methods in Molecular Biology, volume 613, Drug-DNA Interaction Protocols, Editied KR Fox, Chapter 17 Measurement of DNA interstrand Crosslinking in Individual Cells Using the Single Cell Gel Electrophoresis (Comet) Assay. 267-282.

Speit, G., Kojima, H., Burlinson, B., Collins, A.R., Kasper, P., Plappert-Helbig, U., Uno, Y., Vasquez, M., Beevers, C., De Boeck, M., *et al.* (2015). Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT). Mutat Res Genet Toxicol Environ Mutagen *783*, 6-12.

Speit, G., Vasquez, M., and Hartmann, A. (2009). The comet assay as an indicator test for germ cell genotoxicity. Mutation Research *681*, 3-12. .

Suzuki, H., Ikeda, N., Kobayashi, K., Terashima, Y., Shimada, Y., Suzuki, T., Hagiwara, T., Hatakeyama, S., Nagaoka, K., Yoshida, J., *et al.* (2005a). Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats A study by the Collaborative Study group for the Micronucleus Test (CSGMT)/ Japanese Environmental Mutagenicity Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). Mutation Research *583*, 133-145.

Field Code Changed

Suzuki, H., Ikeda, N., Kobayashi, K., Terashima, Y., Shimada, Y., Suzuki, T., Hagiwara, T., Hatakeyama, S., Nagaoka, K., Yoshida, J., *et al.* (2005b). Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). . Mutation Research *583*, 133-145.

Thybaud, V., Aardema, M., Clements, J., Dearfield, K., Galloway, S., Hayashi, M., Jacobson-Kram, D., Kirkland, D., MacGregor, J., Marzin, D., *et al.* (2007). Strategy for genotoxicity testing: Hazard identification and risk assessment in relation *in vitro* testing. Mutation Research *627*, 41-58.

Thybaud, V., Dean, S., Nohmi, T., de Boer, J., Douglas, G., Glickman, B., Gorelick, N., Heddle, J., Heflich, R., Lambert, I., *et al.* (2003). *In vivo* transgenic mutation assays. Mutation Research *540*, 141-151.

Torous, D., Dertinger, S., Hall, N., and Tometsko, C. (2000). Enumeration of micronucleated reticulocytes in rat peripheral blood: a flow cytometric study. Mutation Research *465*, 91-99.

Trivedi, P.P., Kushwaha, S., Tripathu, D.N., and Jena, G.B. (2010). Evaluation of male germ cell toxicity in rats: Correlation between sperm head morphology and sperm comet assay. Mut.Res. *in press*.

Tweats, D.J., Blakey, D., Heflich, R.H., Jacobs, A., Jacobsen, S.D., Morita, T., Nohmi, T., O'Donovan, M.R., Sasaki, Y.F., Sofuni, T. and Tice, R. (2007a). Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests. I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards. Mutation Research *627*, 78-91.

Tweats, D., Blakely, D., Heflich, R., Jacobs, A., Jacobsen, S., Morita, T., Nohmi, T., O'Donovan, M., Sasaki, Y., Sofuni, T., *et al.* (2007b). Report of the IWGT working group on strategy/interpretation for regulatory *in vivo* tests. II. Identification of *in vivo*-only positive compounds in the bone marrow micronucleus test. Mutation Research *627*, 92-105.

UKEMS. (1989). Statistical evaluation of mutagenicity test data. UKEMS sub-committee on guidelines for mutagenicity testing. Report . Part III. Editor Kirkland DJ. Published Cambridge University Press.

Uno, Y., Kojima, H., and Hayashi, M. (2015). The JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay. Mutat Res Genet Toxicol Environ Mutagen *786-788*, 2.

Vasquez, M. (2010). Combining the *in vivo* comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. Mutagenesis *25*, 187-199.

Vogt, E., Kirsch-Volders, M., Parry, J., and Eichenlaub-Ritter, U. (2008). Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mut.Res. *651*, 14-29.

Wakata, A., Matsuoka, A., Yamakage, K., Yoshida, J., Kubo, K., Kobayashi, K., Senjyu, N., Itoh, S., Miyajima, H., Hamada, S., *et al.* (2006). SFTG international collaborative study on *in vitro* micronucleus test IV. Using CHL cells. Mutation Research *607*, 88-124.

Wakata, A., Miyamae, Y., Sato, S., Suzuki, T., Morita, T., Asano, N., Awogi, T., Kondo, K., and Hayashi, M. (1998). Evaluation of the Rat Micronucleus Test with Bone Marrow and peripheral Blood: Summary of the 9th Collaborative Study by the CSGMT/JEMS.MMS. Environ Mol Mutagen *32*, 84-100.

Wang, J., Sawyer, J., Chen, L., Chen, T., Honma, M., Mei, N., and Moore, M. (2009). The Mouse Lymphoma Assay Detects Recombination, Deletion and Aneuploidy. Toxicological Sciences *109*, 96-105.

Westerink, M., Stevenson, J., Horbach, G., Saito, Y., and Schoonen, W. (2010). The development of RAD51C, Cystatin A, p53 and Nrf2 luciferase-reported assays in metabolically competent HepG2 cells for the assessment of mechanism-based genotoxicity and oxidative stress in early research phase of drug development. Mutation Research *696*, 21-40.

Westerink, W., Stevenson, J., Lauwers, A., Griffioen, G., Horbach, G., and Schoonen, W. (2009). Evaluation of the Vitotox™ and RadarScreen assays for rapid assessment of genotoxicity in the early research phase of drug development. Mutation Research *676*, 113-130.

Witt, I., Plappert, U., de Wall, H., and Hartmann, A. (2007). Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation part III. The Comet Assay as an Alternative to *In vitro* Clastogenicity Tests for Early Drug Candidate Selection. Toxicological Sciences *97*, 21-26.

Witt, K., Hughes, L., Burka, L., McFee, A., Mathews, J., Black, S., and Bishop, J. (2003). Mouse bone marrow micronucleus test results do not predict the germ cell mutagenicity of N-hydroymethylacrylamide in the mouse dominant lethal assay. Environ Mol Mutagen *41*, 111-120.

Witt, K., Knapton, A., Wehr, C., Hook, G., Mirsalis, J., Shelby, M., and MacGregor, J. (2000). Micronucleated Erythrocyte Frequency in Peripheral Blood of $B6C3F_1$ Mice from Short-Term, prechronic, and Chronic Studies of the NTP Carcinogensis Bioassay Program. Environmental and Molecular Mutagenesis *36*, 163-194.

Worth, A., Bassan, A., Gallegos, A., Netzeva, T., Patlewicz, G., Pavan, M., Tsakovska, I., and Vracko, M. (2005). The Characterisation of (Quantitative) Structure-Activity Relationships: Preliminary Guidance. Report from European Commission Joint Research Centre, EUR 21866 EN, 2005.

Yauk, C.L., Aardema, M.J., Benthem, J., Bishop, J.B., Dearfield, K.L., DeMarini, D.M., Dubrova, Y.E., Honma, M., Lupski, J.R., Marchetti, F., *et al.* (2015). Approaches for identifying germ cell mutagens: Report of the 2013 IWGT workshop on germ cell assays(). Mutat Res Genet Toxicol Environ Mutagen *783*, 36-54.

Yin, H., Cukrcam, S., Betzendahl, I., Adler, I.-D., and Eichenlaub-Ritter, U. (1998). Trichlorfon exposure, spindle aberratios and nondisjunction in mammalina oocytes. . Chromosoma *107*, 514-522.

Zeiger, E., Ashby, J., Bakale, G., Enslein, K., Klopman, G., and Rosenkranz, H. (1996). Prediction of Salmonella mutagenicity. . Mutagenesis *11*, 471-484.

Commented [BS128]:

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-

Deleted: substance

Deleted:

Deleted: substances

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.

Deleted: substances

Deleted: substances

Field Code Changed

Field Code Changed

Field Code Changed

Field Code Changed

Deleted:

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.

Deleted:
Deleted:
Deleted:
Deleted:
Deleted:
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Deleted:
Deleted: substance
Deleted:

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.

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

Kinetochore staining An immunochemical technique used to detect the presence of centromeric kinetochore proteins in 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

Deleted:
Deleted:
Deleted:
Deleted: substance
Deleted:
Deleted:
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Deleted: micronuclei
Deleted: micronuclei
Deleted: substance

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 ($TK^{+/-}$). 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.

Deleted: micronuclei

Deleted: micronuclei

Deleted: micronuclei

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 polypoidy 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/β-naphthoflavone. S9 is combined with a mix of co-factors which optimize the activity of the mixed function oxidases and form a NADPH generating system which has the capacity to metabolise chemicals *in vitro*.

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

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

Deleted:

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

Deleted:
Deleted:
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Deleted:
Deleted: substance
Deleted:
Deleted: substance
Deleted:
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Deleted:

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.

Deleted:

Deleted: substance

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.

Deleted:

Deleted:

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).

Deleted: