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Biological evaluation of medical devices

Part 33: Guidance on tests to evaluate genotoxicity — Supplement to ISO 10993-3

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National foreword

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary information.

The committee responsible for this document is ISO/TC 194, *Biological and clinical evaluation of medical devices*.

ISO 10993 consists of the following parts, under the general title *Biological evaluation of medical devices*:

— *Part 1: Evaluation and testing within a risk management process*

— *Part 2: Animal welfare requirements*

— *Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity*

— *Part 4: Selection of tests for interactions with blood*

— *Part 5: Tests for in vitro cytotoxicity*

— *Part 6: Tests for local effects after implantation*

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 9: Framework for identification and quantification of potential degradation products*

— *Part 10: Tests for irritation and delayed-type hypersensitivity*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

— *Part 13: Identification and quantification of degradation products from polymeric medical devices*

— *Part 14: Identification and quantification of degradation products from ceramics*

— *Part 15: Identification and quantification of degradation products from metals and alloys*

— *Part 16: Toxicokinetic study design for degradation products and leachables*

— *Part 17: Establishment of allowable limits for leachable substances*

— *Part 18: Chemical characterization of materials*

— *Part 19: Physico-chemical, morphological and topographical characterization of materials (Technical specification)*

— *Part 20: Principles and methods for immunotoxicology testing of medical devices (Technical specification)*

— *Part 33: Guidance on tests to evaluate genotoxicity - Supplement to ISO 10993-3 (Technical Report)*

Introduction

Genotoxicity tests are designed to detect compounds which induce genetic damage directly or indirectly by various mechanisms. These tests should enable hazard identification with respect to genetic damages. Expression of gene mutations, large scale chromosomal damage, recombination, and numerical changes are generally considered to be essential for heritable effects and the multi-step carcinogenesis. A positive genotoxicity test provides an indication that further testing can be warranted to determine the carcinogenic potential of the compound. Because the relationship between exposure to particular chemicals and carcinogenesis is established for man, while a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been used mainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound can induce heritable effects is considered to be just as serious as the suspicion that a compound can induce cancer. In addition, the outcome of such tests can be valuable for the interpretation of carcinogenicity studies.

TECHNICAL REPORT **ISO/TR 10993-33:2015(E)**

Biological evaluation of medical devices —

Part 33: Guidance on tests to evaluate genotoxicity — Supplement to ISO 10993-3

1 Scope

There are differences between the views of regulatory bodies on the subject of genotoxicity testing. The purpose of this Technical Report is to provide background information to facilitate the selection of tests and guidance on the performance of tests.

2 Selection of tests

Since chemicals can induce genetic damage by different mechanisms, a battery of tests sensitive to different types of genetic damage are thought to provide the best assurance for detecting genotoxic hazard. The tests selected usually include tests to detect point mutations and tests to detect chromosomal aberrations. Both bacterial cells and cultured mammalian cells are used to detect genotoxic agents. *in vivo* tests are sometimes incorporated into these test batteries. These tests are sometimes included in the initial test battery or are used to clarify results from *in vitro* tests, see Reference [13].

3 Recommended tests

Although there are some variations in details, the same genotoxicity tests are commonly recommended by most regulatory agencies. The following are commonly recommended tests:

— bacterial reverse mutation test (see OECD 471[1] and Clause 6);

— *in vitro* mammalian chromosome aberration test (see OECD 473[2] and Clause 7);

— *in vitro mammalian micronucleus test* (see OECD 487[6] and Clause 8);

— *in vitro* mammalian cell gene mutation test using mouse lymphoma (L5178Y) cells (see OECD 475[4] and Clause 9);

— *in vivo* mammalian erythrocyte micronucleus test (see OECD 474[3] and Clause 10);

— *in vivo* chromosome aberration test (see OECD 475[5] and Clause 11).

For medical devices, a battery of tests is commonly used for genotoxicity evaluations. The general strategy identified in ISO 10993-3 is as follows:

a) test for gene mutations in bacteria. Bacterial Reverse Mutation Assay, OECD 471[1] technically modified for medical devices to allow, for example, testing with extracts from devices (see Clause 6);

and either

b) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells, Chromosome aberration test, OECD 473[2] technically modified for medical devices (see Clause 7), or

c) an *in vitro* mouse lymphoma tk assay, OECD 476[5] technically modified for medical devices (see Clause 8) including detection of small (slow growing) and large colonies, or

d) an *in vitro* mammalian cell micronucleus test for chromosomal damage and aneugenicity, OECD 487 technically modified for medical devices, (see Clause 8).

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) recommends a three-test battery described in the ICH S2(R1) Genotoxicity, which can be required for medical devices by some regulatory authorities.

4 Use of *in vitro* tests to detect genotoxicity

In vitro tests are commonly used for identifying the potential of chemicals to induce genotoxicity. Multiple tests are used because no single test detects all known genotoxins. Genotoxins often lead to different effects (e. g. large scale or chromosomal damage vs. small scale damage or point mutations or different DNA sequence specificity). Also, the resulting genetic damage has differing susceptibility to DNA repair. The "ICH test battery" was developed to cast a wider net for detecting genotoxins. Although *in vitro* genotoxicity tests can be considered overly sensitive, these tests detect most rodent genotoxic carcinogens. Comparisons of the "scorecards" of genotoxicity assay with those of rodent carcinogenicity assays have found that the *in vitro* mammalian assays generated a number of "false positives" (i.e. agents testing positive that were not rodent carcinogens). However, it is not clear that the rodent carcinogenicity assay is the appropriate standard, rather than detection of genotoxicity per se.

Later work identified two new classes of pharmaceuticals causing DNA damage by interference with topoisomerases. These are responsible for substantial numbers of the *in vitro* false positives, see Reference [29]. Later work indicated much lower percentages of unexplained *in vitro* positive results with pharmaceuticals, see Reference [16]. Unfortunately, all of the information on the ability of genotoxicity to predict carcinogenicity and germ cell mutagenicity was developed from the analysis of industrial chemicals and pharmaceuticals. Medical device testing usually includes the use of extracts, which often contain complex mixtures of chemicals. Although future effects are unknown, device extracts have generated limited number of positives with unknown constituents to date.

5 Use of *in vivo* tests to detect genotoxicity

The *in vivo* genotoxicity tests are an integral part of the ICH test battery and are used in a weight of evidence approach in the evaluation of pharmaceuticals. For these tests, a demonstration that the chemical or its metabolite has reached the target organ is required. For medical devices, the latter requirement is often difficult to fulfil since complex mixtures are usually tested and the dose of agent(s) in extracts can be below the detection level of the system.

The *in vivo* test for chromosomal damage using rodent haematopoietic cells is included in the test battery to provide additional relevant factors (absorption, distribution, metabolism, excretion) that can influence the genetic activity of chemicals, see Reference [14]. There are also a small number of genotoxic carcinogens that are reliably detected by the *in vivo* bone marrow tests for chromosomal damage that have yielded negative/weak/conflicting results in the pairs of *in vitro* tests outlined in the standard battery options (e.g. bacterial reverse mutation plus one of a selection of possible tests with cytogenetic evaluation of chromosomal damage or bacterial mutation plus the mouse lymphoma tk assay). A few industrial chemical carcinogens such as urethane and benzene fall into this category, see Reference [31].

The value of including *in vivo* tests as part of the initial genotoxicity assessment is controversial. The limited sensitivity of *in vivo* tests to detect a significant number of carcinogens (see Reference [10] and Reference [27]) can argue against their use. However, the concern that a small group of biologically active compounds that are known or suspected human carcinogens cannot be easily detected by *in vitro* tests (see Reference [26]) argues for their use in circumstances where the extent of exposure to biologically active constituents of a medical device indicates the need for greater reassurance.

6 Bacterial reverse mutation assay

6.1 General

The following procedure for the bacterial reverse mutation assay was adapted for medical devices from OECD 471.[1] For evaluation of genotoxic potential of medical devices, medical device material, extracts or extracted and evaporated residues can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Suspensions of bacterial cells are exposed to the test sample in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after 48 h or 72 h of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

6.2 Preparations

6.2.1 Bacteria

Cultures of bacteria in late exponential growth or early stationary phase of growth (approximately 10^9 cells/ml) should be used.

The recommended culture temperature is 37 °C.

The recommended combination of strains is

— *S. typhimurium* TA1535, and

— *S. typhimurium* TA1537 or TA97 or TA97a, and

— *S. typhimurium* TA98, and

— *S. typhimurium* TA100, and

— *E. coli* WP2 *uvrA*, or *E. coli* WP2 *uvrA* (pKM101), or *S. typhimurium* TA102.

Established procedures for stock culture preparation, marker verification, and storage should be used.

The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains and tryptophan for *E. coli* strains).

The following phenotypic characteristics should be checked:

a) presence or absence of R-factor plasmids, where appropriate:

   1) ampicillin resistance in strains TA98, TA100, and TA97a or TA97 and WP2 *uvrA* (pKM101);

   2) ampicillin + tetracycline resistance in strain TA102;

b) the presence of characteristic mutations:

   1) rfa mutation in *S. typhimurium* through sensitivity to crystal violet;

   2) *uvrA* mutation in *E. coli* or *uvrB* mutation in *S. typhimurium*, through sensitivity to ultraviolet light.

The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

6.2.2 Medium

An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, is used.

6.2.3 Metabolic activation

Bacteria should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction S9 prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range from 5 % volume fraction to 10 % volume fraction in the S9 mix.

The supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded. If the S9 is an in-house source, then source and method of preparation should be documented. Regardless, the S9 activity should be verified using two reference promutagens in a defined strain (e.g. *S. typhimurium* TA100) and compared to the historical control.

The concentration of S9 homogenate should be expressed as activity units per plate since different suppliers can prepare S9 differently, e.g. use different co-factors in S9 mix, different ratio of tissue to homogenizing fluid during S9 preparation.

The buffer and component concentrations should be defined.

Simple omission of the S9 mix component in the top agar is not recommended in the absence of metabolic activation system, as the differing volumes of the agar overlay will alter the perceived dose of compound (at least initially, depending on solubility and/or diffusion into the basal agar). The S9 mix should be replaced with an appropriate buffer.

6.2.4 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (see ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (see ISO 10993-3, Annex A, Method B and Method C).

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent adsorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

6.3 Test conditions

6.3.1 Solvents

The test solvent should be selected in accordance with ISO 10993-12 or ISO 10993-3, Annex A, and should be compatible with the survival of the bacteria and the S9 activity. Rationale for solvent selection should be documented. If the selected solvent has not been commonly used, evidence/data demonstrating compatibility should be presented. If other than well-known solvents are used, their inclusion should be supported by data indicating their compatibility.

6.3.2 Exposure concentrations

The maximum test concentrations will depend on the solubility and cytotoxicity of the test compound or the cytotoxicity of the test sample extract.

Dose Range Finding Study (DRF study)

A DRF study may be conducted prior to the main study if cytotoxicity of the test sample is expected to be significant, e.g. cytotoxicity or growth inhibition greater than 50 %.

Cytotoxicity can be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a test sample can be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye.

Limit Study

For soluble, non-cytotoxic test compounds (determined in the DRF study), a single test at one dose level of at least 5 mg/plate or 5 µl/plate (see ISO 10993-3, Annex A, Method A or, if feasible, Method B) or 0,1ml of a 100 % (neat) test sample extract (see ISO 10993-3, Annex A, Method C) is acceptable. For test articles prepared following guidance provided in ISO 10993-12, in most situations, a limit study using 100 % test sample extract is acceptable and no further dosing study is necessary.

Main Dosing Study

If the test sample shows visible signs of precipitation or is cytotoxic already below dose level of 5 mg/plate or 5 µl/plate or 100 % of a test sample extract, a full study with at least five different analysable concentrations of the test sample should be used. For cytotoxic test compounds/test sample extracts, the dose levels for revertant frequency should cover a range from the maximum to little or no cytotoxicity. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment. The precipitate should not interfere with scoring.

Criteria to be taken into consideration when determining the highest amount of test sample to be used should include cytotoxicity and solubility in the final treatment mixture. It can be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity might be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a test sample can be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration will depend on the test sample preparation method selected.

a) For soluble non-cytotoxic test samples (see ISO 10993-3, Annex A, Method A), the recommended maximum test concentration is 5 mg/plate or 5 µl/plate. For non-cytotoxic test samples that are not soluble at 5 mg/plate or 5 µl/plate (in case of liquid chemicals), one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/plate or 5 µl/plate (in case of liquid chemicals) should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

b) For test samples in accordance with ISO 10993-3, Annex A, Method B, the recommended maximum test concentration is 5 mg/plate, if feasible. For non-cytotoxic test samples that are not soluble at 5 mg/plate, one or more concentrations tested should be insoluble in the final treatment mixture. The precipitate should not interfere with the scoring. Test samples that are cytotoxic below 5 mg/plate should be tested up to a cytotoxic concentration.

c) For test samples in accordance with ISO 10993-3, Annex A, Method C, the recommended maximum test concentration is 100 % of the test sample extract.

When a precipitate is observed or the test sample is cytotoxic, at least five different analysable concentrations of the test sample should be used. For preliminary experiments, test concentrations using

half log intervals can be helpful. Smaller intervals can be appropriate when subsequent experiments are performed. If the test sample is soluble and not cytotoxic, a single maximum concentration is acceptable.

6.3.3 Controls

Concurrent positive and negative (vehicle) controls (both with and without metabolic activation) should be included for each strain. Except for the treatment with the test sample preparation, the positive and negative control groups should be processed in an identical manner as the treatment groups.

A viable bacterial count on the stock culture should be demonstrated and recorded as part of each assay.

The induced mutation rates with the reference mutagen should be within the range reported in the literature.

For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

— 9,10-Dimethylanthracene [CAS no. 781-43-1];

— 7,12-Dimethylbenzanthracene [CAS no. 57-97-6];

— Congo Red [CAS no. 573-58-0] (for the reductive metabolic activation method);

— Benzo(a)pyrene [CAS no. 50-32-8];

— Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)];

— 2-Aminoanthracene [CAS no. 613-13-8].

2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9 mix. If 2-Aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, e.g. benzo(a)pyrene, dimethylbenzanthracene.

For assays performed without metabolic activation system, examples of strain-specific positive controls are given in Table 1.

Table 1 — Examples of strain-specific positive controls

Chemical	CAS No.	Strain
Sodium azide	26628-22-8	TA1535 and TA100
2-Nitrofluorene	607-57-8	TA98
9-Aminoacridine or ICR191	17070-45-0 90-45-9	TA1537, TA97, and TA97a
Cumene hydroperoxide Or Methyl methanesulfonate	80-15-9 66-27-3	TA102
Mitomycin C	50-07-7	WP2 *uvrA* and TA102
N-Ethyl-*N*'-nitro-*N*-nitrosoguanididine or 4-Nitroquinoline 1-oxide or *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine	4245-77-6 56-57-5 70-25-7	WP2, WP2 *uvrA,* and WP2 *uvrA* (pKM101)
NOTE Other appropriate positive control reference substances may be used.		

Table 1 *(continued)*

Chemical	CAS No.	Strain
Furylfuramide (AF-2)	3688-53-7	WP2 *uvrA* plasmid-containing strains
NOTE Other appropriate positive control reference substances may be used.		

6.4 Procedure

6.4.1 Treatment with test sample

For the plate incorporation method, without metabolic activation, usually 0,1 ml of the test sample, 0,1 ml of fresh bacterial culture (containing at least 10^8 viable cells) and 0,5 ml of sterile buffer are mixed with 2,0 ml of overlay agar. For the assay with metabolic activation, usually 0,5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 % to 10 % volume fraction in the metabolic activation mixture) are mixed with the overlay agar (2,0 ml), together with the bacteria and test sample/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

For the preincubation method, the test sample/test solution is preincubated with the test strain (containing at least 10^8 viable cells) and sterile buffer or the metabolic activation system (0,5 ml) usually for 20 min or more at 37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0,1 ml of test sample or extract, 0,1 ml of bacteria, and 0,5 ml of S9 mix or sterile buffer, are mixed with 2,0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

For an adequate estimate of variation, triplicate plating should be used at each dose level.

The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels.

If quantitative comparisons are to be made between experiments carried out in the presence and absence of S9 mix, the experiments should be performed on the same day.

For *in vitro* assays with built-in confirmatory elements such as multiple treatment lengths or tests with and without metabolic activation, further confirmatory testing in the case of clearly negative or positive test results is not usually needed. Equivocal results can require repeating tests, possibly with a modified protocol such as appropriate spacing of the test concentrations.

6.4.2 Incubation

All plates in a given assay should be incubated at 37 °C for 48 h to 72 h. After the incubation period, the number of revertant colonies per plate is counted.

6.4.3 Data collection

Automated colony counters should be calibrated against a series of authentic hand-counted plates encompassing a range of mutant colonies, from very low to very high counts and colonies of varying sizes.

The condition of the bacterial background for evidence of test article extract toxicity should be evaluated by using a dissecting microscope or darkfield counter. The precipitate should be evaluated by visual examination without magnification. Toxicity and degree of precipitation should be scored relative to the corresponding extraction blank using the standardized method (e.g. table at the end of document).

6.5 Data and reporting

6.5.1 Treatment of results

Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control and untreated control, if used) and positive control plates should also be given.

Individual plate counts, the mean number of revertant colonies per plate, and the standard deviation should be presented for the test sample and positive and negative (untreated and/or solvent) controls.

For *in vitro* assays with built-in confirmatory elements such as multiple treatment lengths or tests with and without metabolic activation, further confirmatory testing in the case of clearly negative or positive test results is not usually needed. Equivocal results can require repeating tests, possibly with a modified protocol such as appropriate spacing of the test concentrations.

For ISO 10993-3, Annex A, Method A and Method B, the dose of the test compound should be expressed by mass (per plate or per ml) and not by volume.

6.5.2 Evaluation and interpretation of results

There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results.

A test substance for which the results do not meet the above criteria is considered nonmutagenic in this test.

Although most experiments will give clearly positive or negative results, the data set will preclude making a definite judgement about the activity of the test sample in rare cases. Results can remain equivocal or questionable regardless of the number of times the experiment is repeated. An equivocal response is an increase in a revertant count that partially meets the criteria for evaluation as positive. This could be an increase that does not meet the threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions or frame shifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test sample is not mutagenic in the tested species.

6.5.3 Criteria for a valid test

The following criteria should be met for the mutagenicity assay of each test article extract to be considered valid. All *Salmonella* tester strain cultures recommended above should demonstrate the presence of the deep rough mutation (rfa) and the deletion in the *uvrB* gene. Cultures of tester strains TA98 and TA100 should demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvrA* cultures should demonstrate UV sensitivity caused by the *uvrA* mutation. All cultures should demonstrate the characteristic mean number of spontaneous revertants in the extraction blanks. For example, TA98, 10 to 50; TA100, 80 to 240; TA1535, 5 to 45; TA1537, 3 to 21; WP2 *uvrA*, 10 to 60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titres should be greater than or equal to $0{,}3 \times 10^9$ cells/ml. The mean of the number of revertants in each positive control should exhibit significant increase comparing with that in the respective solvent control and within the historical control range in each laboratory.

When the test extract is toxic, the highest dose level should meet one or both of the following criteria:

— reduction greater than 50 % with dose-dependency in the mean number of revertants per plate as compared to the mean corresponding solvent control;

— at least a moderate reduction in the background lawn (see code 3, 4, or 5 in Table 2).

Table 2 — Background lawn observation code for bacterial mutation tests

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the extraction blank.
3	Moderately reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the extraction blank.
4	Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the extraction blank such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over more than or equal to 90 % of the plate.
6[a]	Obscured by particulates	The background bacterial lawn cannot be accurately evaluated due to microscopic test article particulate.
7[a]	Non-interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10 % of the revertant colony count (e.g. less than or equal to three particles on a plate with 30 revertants).
8	Interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total more than 10 % of the revertant colony count (e.g. more than three particles on a plate with 30 revertants).

[a] Nanomaterial or nanoparticles are not included in the definition of precipitates and particles.

6.5.4 Test report

The test report should include the following information:

a) test sample:

   1) medical device, components, or materials tested;

   2) identification data and CAS No., if known;

   3) physical nature and purity of materials tested, if known;

   4) physicochemical properties relevant to the conduct of the study;

   5) stability of the test sample, if known;

   6) sterilization status,;

   7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

   8) description of the test sample physical characteristics (e.g. clarity, colour, and presence of particulates);

   9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

10) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

b) solvent/vehicle:

1) justification for choice of solvent/vehicle;

2) solubility and stability of the test sample in solvent/vehicle, if known;

c) strains:

1) strains used;

2) number of cells per culture;

3) strain characteristics;

d) test conditions:

1) amount of test sample per plate (mg/plate or µl/plate) with rationale for selection of dose and number of plates per concentration;

2) media used;

3) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

4) type and composition of metabolic activation system, including acceptability criteria;

5) treatment procedures;

e) results:

1) signs of toxicity;

2) signs of precipitation;

3) individual plate counts;

4) mean number of revertant colonies per plate and standard deviation;

5) dose-response relationship, where possible;

6) statistical analyses, if any;

7) historical negative (solvent/vehicle) and positive control data, with e.g. ranges, means, and standard deviations;

f) record the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.). If the S9 is an in-house source, then source and method of preparation should be documented;

g) include S9 concentration used in assay;

h) record test strain titre results;

i) protocol deviations and a discussion on how the deviations affect the study results. State if no deviation;

j) discussion of the results;

k) conclusion.

7 *In vitro* mammalian chromosome aberration test

7.1 General

The following procedure for the *in vitro* mammalian chromosome aberration test was adopted for medical devices using OECD 473.[2] For evaluation of genotoxic potential of medical devices, medical device material, extracts or extracted and evaporated residues can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Cell cultures are exposed to the test sample both with and without metabolic activation. At predetermined intervals (see 7.4.1) after exposure of cell cultures to the test sample, they are treated with a metaphase-arresting substance (e.g. Colcemid®[1)] or colchicine), harvested, stained, and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

7.2 Preparations

7.2.1 Cells

A variety of cell lines, strains, or primary cell cultures, including human cells, may be used (e.g. Chinese hamster CHO, V79 and CHL cell lines, human or other mammalian peripheral blood lymphocytes).

7.2.2 Media and culture conditions

Appropriate culture media and incubation conditions (culture vessels, CO_2 concentration, temperature, and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

7.2.3 Preparation of cultures

Established cell lines and strains

Cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37 °C.

Lymphocytes

Whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes obtained from healthy subjects are added to culture medium containing a mitogen (e.g. phytohemagglutinin) and incubated at 37 °C.

7.2.4 Metabolic activation

Cells should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor supplemented post-mitochondrial fraction S9 prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range between 1 % and 10 % volume fraction in the final test medium. The condition of a metabolic activation system can depend upon the class of chemical being tested. In some cases, it can be appropriate to utilize more than one concentration of post-mitochondrial fraction.

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1) Colcemid is the trade name of a product supplied by Ciba-Geigy Company, Basel, Switzerland. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Record the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, metabolic activity, etc.). If the S9 is an in-house source, then source and method of preparation should be documented.

The buffer and component concentrations should be recorded.

Simple omission of the S9 mix component is not recommended, as the differing volumes will alter the perceived dose of compound. The S9 mix should be replaced with an appropriate buffer.

7.2.5 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (See ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (See ISO 10993-3, Annex A, Method B and Method C).

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

7.3 Test conditions

7.3.1 Solvents

The test solvents are selected in accordance with ISO 10993-12 or ISO 10993-3, Annex A and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvents are used, their inclusion should be supported by data indicating their compatibility.

7.3.2 Exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system, and changes in pH or osmolality.

The recommended maximum test concentration will depend on the test sample preparation method selected. For ISO 10993-12, a limit study using 100 % test sample extract is acceptable and no further dosing study is necessary. For ISO 10993-3 Annex A, the following should be considered.

a) For soluble non-cytotoxic test samples (ISO 10993-3, Annex A, Method A), the recommended maximum test concentration is 5 mg/ml or 5 µl/ml. For non-cytotoxic test samples that are not soluble at 5 mg/ml or 5 µl/ml, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml or 5 µl/ml should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

b) For test samples in accordance with ISO 10993-3, Annex A, Method B, the recommended maximum test concentration is 5 mg/ml, if feasible. For non-cytotoxic test samples that are not soluble at 5 mg/ml, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

c) For test samples in accordance with ISO 10993-3, Annex A, Method C, the recommended maximum test concentration is 100 % of the test sample extract. For non-cytotoxic test samples that are not soluble at 100 %, one or more concentrations tested should be insoluble in the final treatment

mixture. Test samples that are cytotoxic already below 100 % should be tested up to a cytotoxic concentration.

Replacement of medium with fresh medium plus extract is preferable to avoid nutrient depletion.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as relative increase in cell counts (RICC) or relative population doubling (RPD), degree of confluency, viable cell counts, or mitotic index. It can be useful to determine cytotoxicity and solubility in a preliminary experiment.

Where cytotoxicity occurs, at least three analysable concentrations should be used. These concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentrations should be separated by no more than a factor between 2 and the square root of 10. At the time of harvesting, the highest concentration should show a significant reduction in population doubling, cell count, or mitotic index (all greater than 50 % when compared to negative/solvent control). The mitotic index is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment. However, the mitotic index is acceptable for suspension cultures in which other toxicity measurements can be cumbersome and impractical.

For relatively non-cytotoxic extracts, the neat extracts should be applied at 10 % (aqueous solutions) or 1 % (non-aqueous/solvents) of the culture volume. If cell culture medium is used as extraction vehicle, the recommended maximum test concentration is 100 % of the test sample extract. If culture medium without serum is used as a polar solvent for extraction, the test extract is tested neat following supplementation with serum before dosing the cells. The test extract in the cell culture medium with serum (as a non-polar solvent) is tested as neat extract. Test samples that are cytotoxic already below 100 % should be tested up to the appropriate cytotoxicity concentration required for the test. For relatively noncytotoxic test samples, the maximum concentration should be 5 µl/ml (in case of pure liquid chemicals), 5 mg/ml, or 0,01 M, whichever is the lowest. Under this circumstance, a single maximum concentration is acceptable.

For relatively insoluble test samples that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration), it is advisable to test at more than one concentration with visible precipitation. It can be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum, etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

7.3.3 Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a clastogenic response.

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background which demonstrates the sensitivity of the test system. Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader.

Examples of positive control substances include the following:

a) absence of exogenous metabolic activation:

   1) Methyl methanesulphonate [CAS no. 66-27-3];

   2) Ethyl methanesulphonate [CAS no. 62-50-0];

   3) Mitomycin C [CAS no. 50-07-7];

4) 4-Nitroquinoline-*N*-Oxide [CAS no. 56-57-5];

b) presence of exogenous metabolic activation:

1) Benzo(a)pyrene [CAS no. 50-32-8];

2) Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)].

Other appropriate positive control substances may be used. The use of chemical class related positive control chemicals may be considered, when available.

Negative controls consisting of solvent or vehicle alone in the treatment medium and treated in the same way as the treatment cultures should be included for every harvest time. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or clastogenic effects are induced by the chosen solvent.

7.4 Procedure

7.4.1 Treatment with test sample or extract and harvest time

In the first experiment, cells should be exposed to the test sample both with and without metabolic activation for 3 h to 6 h and sampled at a time equivalent to about 1,5 normal cell cycle length after the beginning of treatment. If this protocol gives negative results both with and without activation, an additional experiment without activation should be done with continuous treatment until sampling at a time equivalent to about 1,5 normal cell cycle lengths. Certain chemicals can be more readily detected by treatment/sampling times longer than 1,5 cycle lengths. Negative results with metabolic activation need to be confirmed on a case-by-case basis. When both polar and non-polar extracts are tested, negative results with both extracts are considered confirmation. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Proliferating cells are treated with the test sample in the presence and absence of a metabolic activation system. Treatment of lymphocytes should commence at about 48 h after mitogenic stimulation.

Duplicate cultures should normally be used at each concentration and are strongly recommended for negative/solvent control cultures.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels.

7.4.2 Chromosome preparation

Cell cultures are treated with Colcemid®[2)] or colchicine usually one hour to three hours prior to harvesting. Each cell culture is harvested and processed separately for the preparation of chromosomes. Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

7.4.3 Analysis

When the mitotic index is scored, a minimum of 1 000 cells per culture should be evaluated.

All slides, including those of positive and negative controls, should be independently coded and randomized before microscopic analysis. Since fixation procedures often result in the breakage of a proportion of metaphase cells with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the modal number ±2 for all cell types. At least 200 well-spread metaphases should be scored per concentration and control equally divided amongst the duplicates, if applicable. This number can be reduced when high numbers of aberrations are observed.

Though the purpose of the test is to detect structural chromosome aberrations, it is important to record polyploidy and endoreduplication when these events are seen.

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2) Colcemid is the trade name of a product supplied by Ciba-Geigy Company, Basel, Switzerland. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

7.5 Data and reporting

7.5.1 Treatment of results

The experimental unit is the cell and, therefore, the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiment(s) should also be recorded.

Individual culture data should be provided. Additionally, all data should be summarized in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. The need to confirm negative results has been discussed previously. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

If, having selected a cell under low power, it is obvious under high power that the quality of the metaphase precludes accurate CA scoring (e.g. excessively "fuzzy" or overlapping chromosomes), it should not be included in the scoring. Initially, a minimum of 200 cells should be scored from each treatment group, 100 from each of two replicates. If ambiguous results are obtained, further "blind" scoring of these same samples may be undertaken, including the additional replicates of negative or solvent controls.

7.5.2 Evaluation and interpretation of results

There are several criteria for determining a positive result, such as a reproducible increase in the number of cells with chromosome aberrations. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response.

An increase in the number of polyploid cells can indicate that the test sample has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations. An increase in the number of cells with endoreduplicated chromosomes can indicate that the test sample has the potential to inhibit cell cycle progression. See Reference [7] and Reference [8].

A test sample or extract for which the results do not meet the above criteria is considered nonmutagenic in this system.

Although most experiments will give clearly positive or negative results, the data set will preclude making a definite judgement about the activity of the test samples in rare cases. Results can remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* chromosome aberration test indicate that the test sample induces structural chromosome aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test sample does not induce chromosome aberrations in cultured mammalian somatic cells.

7.5.3 Test report

The test report should include the following information:

a) test sample:

   1) medical device, components, or materials tested;

   2) identification data and CAS no., if known;

   3) physical nature and purity of materials tested, if known;

4) physicochemical properties relevant to the conduct of the study;

5) stability of the test sample, if known;

6) sterilization status;

7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

8) description of the test sample physical characteristics (e.g. clarity, colour, and presence of particulates);

9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

10) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

b) solvent/vehicle:

1) justification for choice of solvent/vehicle;

2) solubility and stability of the test sample in solvent/vehicle, if known;

c) cells:

1) type and source of cells;

2) karyotype features and suitability of the cell type used;

3) absence of mycoplasma, if applicable;

4) information on cell cycle length;

5) sex of blood donors, whole blood or separated lymphocytes, mitogen used;

6) number of passages, if applicable;

7) methods for maintenance of cell cultures, if applicable;

8) modal number of chromosomes;

d) test conditions:

1) identity of metaphase arresting substance, its concentration, and duration of cell exposure;

2) test sample preparation method with rationale for selection of method;

3) rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;

4) composition of media, CO_2 concentration, if applicable;

5) concentration of test sample or extract;

6) volume of vehicle and test sample or extract added;

7) incubation temperature;

8) incubation time;

9) duration of treatment;

10) cell density at seeding, if appropriate;

11) the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded. If the S9 is an in-house source, then source and method of preparation should be documented;

12) type and composition of metabolic activation system, including acceptability criteria;

13) positive and negative controls;

14) methods of slide preparation;

15) criteria for scoring aberrations;

16) number of metaphases analysed;

17) methods for the measurements of toxicity;

18) criteria for considering studies as positive, negative or equivocal;

e) results:

1) signs of toxicity, e.g. population doubling, cell cycle data, cell counts, mitotic index;

2) signs of precipitation;

3) data on pH and osmolality of the treatment medium, if determined;

4) definition for aberrations, including gaps;

5) number of cells with chromosome aberrations and type of chromosome aberrations, given separately for each treated and control culture;

6) changes in polyploidy, if seen;

7) dose-response relationship, where possible;

8) statistical analyses, if any;

9) concurrent negative (solvent/vehicle) and positive control data;

10) historical negative (solvent/vehicle) and positive control data, with ranges, means, and standard deviations;

f) discussion of the results;

g) conclusion.

8 *In vitro* mammalian micronucleus test

8.1 General

The following procedure for the *in vitro* mammalian micronucleus test was adopted for medical devices using OECD 487.[6] For evaluation of genotoxic potential of medical devices, medical device/material or extracts from medical device/material or extracted and evaporated residues from medical device/materials or chemicals can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Cell cultures are exposed to the test sample both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test sample, the cells are harvested, stained, and cells are analysed microscopically for the presence of micronuclei.

8.2 Preparations

8.2.1 Cells

A variety of cell lines, strains, or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human, or other mammalian peripheral blood lymphocytes).

8.2.2 Media and culture conditions

Appropriate culture media and incubation conditions (culture vessels, CO_2 concentration, temperature, and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

8.2.3 Preparation of cultures

Established cell lines and strains

Cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37 °C.

Lymphocytes

Whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes obtained from healthy subjects are added to culture medium containing a mitogen (e.g. phytohemagglutinin) and incubated at 37 °C.

8.2.4 Metabolic activation

Cells should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor supplemented post-mitochondrial fraction S9 prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range between 1 % and 10 % V/V in the final test medium. The condition of a metabolic activation system can depend upon the class of chemical being tested. In some cases, it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, can provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test sample).

Record the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.). If the S9 is an in-house source, then source and method of preparation should be documented.

The buffer and component concentrations should be recorded.

Simple omission of the S9 mix component is not recommended, as the differing volumes will alter the perceived dose of compound. The S9 mix should be replaced with an appropriate buffer.

8.2.5 Use of cytoB as a cytokinesis blocker

One of the most important considerations in the performance of the micronucleus test *in vitro* is ensuring that the cells being scored have completed mitosis during treatment or post-treatment incubation period, if one is used. CytoB is an agent that has been most widely used to block cytokinesis because it inhibits actin assembly and thus, prevents separation of daughter cells after mitosis, leading to the formation of binucleated cells. CytoB should be used when human lymphocytes are used because cell cycle time will

be variable within cultures and among donors. Other methods have been used when testing adherent cell lines to determine if the cells being divided.

8.2.6 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (See ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (See ISO 10993-3, Annex A, Method B and Method C).

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

8.3 Test conditions

8.3.1 Solvents

The test solvents are selected in accordance with ISO 10993-3, Annex A, or ISO 10993-12 and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvents are used, their inclusion should be supported by data indicating their compatibility.

8.3.2 Exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system, and changes in pH or osmolality.

The recommended maximum test concentration will depend on the test sample preparation method selected. For ISO 10993-12, a limit study using 100 % test sample extract is acceptable and no further dosing study is necessary. For ISO 10993-3, Annex A, the following should be considered.

a) For soluble non-cytotoxic test samples (ISO 10993-3, Annex A, Method A), the recommended maximum test concentration is 5 mg/ml or 5 µl/ml. For non-cytotoxic test samples that are not soluble at 5 mg/ml or 5 µl/ml, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml or 5 µl/ml should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

b) For test samples in accordance with ISO 10993-3, Annex A, Method B, the recommended maximum test concentration is 5 mg/ml, if feasible. For non-cytotoxic test samples that are not soluble at 5 mg/ml, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

c) For test samples in accordance with ISO 10993-3, Annex A, Method C, the recommended maximum test concentration is 100 % of the test sample extract. For non-cytotoxic test samples that are not soluble at 100 %, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 100 % should be tested up to a cytotoxic concentration.

For dosing the cells, the maintenance medium will be replaced with the medium containing the test sample or test extract at appropriate concentration.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, using the relative increase in cell counts (RICC) or relative population doubling (RPD) unless cytoB is used. When cytoB is used, cytotoxicity can be determined using the replication index (RI). Treatment of cultures with cytoB and measurement of the relative frequencies of mononucleate, binucleate, and multi-nucleate cells in the culture, provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity of a treatment and ensures that only cells that divided during or after treatment are scored (Cytokinesis-Block-Proliferation-Index, CBPI).

Where cytotoxicity occurs, at least three analysable concentrations should be used. These concentrations should cover a range from the maximum to little or no toxicity. This will usually mean that the concentrations should be separated by no more than a factor between 2 and the square root of 10. At the time of harvesting, the highest concentration should show a significant reduction in degree of the cytotoxicity parameters mentioned above, (55 ± 5) % when compared to negative /solvent control.

For relatively non-cytotoxic extracts, the neat extracts should be applied at 10 % (aqueous solutions) or 1 % (non-aqueous/solvents) of the culture volume. For relatively noncytotoxic test samples, the maximum concentration should be 5 µl/ml (in case of pure liquid chemicals), 5 mg/ml, or 0,01 M, whichever is the lowest. Under this circumstance, a single maximum concentration is acceptable.

For relatively insoluble test samples that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration), it is advisable to test at more than one concentration with visible precipitation. It can be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum, etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

8.3.3 Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a positive response.

Positive controls are needed to demonstrate the ability to identify clastogens and aneugens at exposure levels expected to give a reproducible and detectable increase over background, demonstrating the sensitivity of the test system, and to affirm the metabolic capability of the S9 preparation. Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include the following:

a) clastogens active without metabolic activation:

   1) Cytosine arabinoside [CAS no. 147-94-4];

   2) Mitomycin C [CAS no. 50-07-7];

b) clastogens requiring metabolic activation:

   1) Benzo(a)pyrene [CAS no. 50-32-8];

   2) Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)];

c) aneugens:

   1) Colchicine [CAS no. 64-86-8];

   2) Vinblastine [CAS no. 143-67-9].

Other appropriate positive control substances may be used. The use of chemical class related positive control chemicals can be considered, when available.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included for every harvest time. In addition, untreated controls should also be used unless there are historical control data demonstrating that no aneugenic or clastogenic effects are induced by the chosen solvent.

8.4 Procedure

8.4.1 Treatment with test sample or extract and harvest time

In the first experiment, cells should be exposed to the test sample both with and without metabolic activation for 3 h to 6 h, followed by removal of the test sample and a growth period of 1,5 to 2 cell cycles. Cells are sampled at a time equivalent to about 1,5 to 2,0 times the normal (untreated) cell cycle length either after the beginning or at the end of treatment. Sampling or recovery times may be extended if it is known or suspected that the test sample affects the cell cycling time.

If this protocol gives negative results both with and without activation, an additional experiment without activation should be done with continuous treatment until sampling at a time equivalent to about 1,5 to 2 normal cell cycle lengths. The suggested treatment schedules are given in Table 3.

Table 3 — Suggest treatment schedules

Cells	+S9	-S9 (Short exposure)	-S9 (Extended exposure)	
Lymphocytes primary cells and cell lines treated with cytoB	– Treat for 3 h to 6 h in the presence of S9. – Remove the S9 and treatment medium. – Add fresh medium and cytoB. – Harvest 1,5 to 2,0 normal cell cycle later.	– Treat for 3 h to 6 h. – Remove the treatment medium. – Add fresh medium and cytoB. – Harvest 1,5 to 2,0 normal cell cycle later.	**Option A:** – Treat for 1,5 to 2,0 normal cell cycles in the presence of cytoB. – Harvest at the end of the exposure period.	**Option B:** – Treat for 1,5 to 2,0 normal cell cycles. – Remove the test sample. – Add fresh medium and cytoB. – Harvest 1,5 to 2,0 normal cell cycle later.
Cell lines treated without cytoB	– Treat for 3 h to 6 h in the presence of S9. – Remove the S9 and treatment medium. – Add fresh medium. – Harvest 1,5 to 2,0 normal cell cycle later.	– Treat for 3 h to 6 h. – Remove the treatment medium. – Add fresh medium. – Harvest 1,5 to 2,0 normal cell cycle later.	**Option A:** – Treat for 1,5 to 2,0 normal cell cycles. – Harvest at the end of the exposure period.	**Option B:** – Treat for 1,5 to 2,0 normal cell cycles. – Remove the test sample. – Add fresh medium. – Harvest 1,5 to 2,0 normal cell cycle later.

Duplicate cultures should normally be used at each concentration and are strongly recommended for negative/solvent control cultures.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels.

8.4.2 Cell harvest and slide preparation

Cell preparation can involve hypotonic treatment, but this step is not necessary if adequate cell spreading can be guaranteed. Cell cytoplasm should be retained to allow the detection of micronuclei and (in the cytokinesis block method) reliable identification of binucleate cells.

The slides can be stained with various methods, such as Giemsa or fluorescent DNA specific dyes, where artefacts can be eliminated, obtained by using non-DNA specific stain. Anti-kinetochore antibodies,

FISH with pancentromeric DNA probes, or primed *in situ* labelling with pancentromer-specific primers may be used to identify the contents (chromosome/chromosomal fragment) of micronuclei.

8.4.3 Analysis

All the slides of the study will be independently coded before the microscopic analysis. Alternatively, coded samples can be analysed using a validated automated flow cytometric or image analysis system.

In cytoB-treated cultures, micronucleus frequencies should be analysed in at least 2 000 binucleated cells per concentration (at least 1 000 binucleated cells per culture, two cultures per concentration) If single cultures are used, at least 2 000 binucleated cells per concentration should be scored from that culture. If substantially less than 1 000 binucleated cells per culture or 2 000, if a single culture is used, are available for scoring at each concentration and if a significant increase in micronuclei is not detected, the test should be repeated using more cells or at less toxic concentrations, whichever is appropriate. Care should be taken not to score binucleate cells with irregular shapes or where the two nuclei differ greatly in size; neither should binucleate cells be confused with poorly spread multi-nucleate cells. Cells containing more than two main nuclei should not be analysed for micronuclei, as the baseline micronucleus frequency can be higher in these cells. Scoring of mononucleate cells is acceptable if the test substance is shown to interfere with cytoB activity.

In cell lines assays without cytoB treatment, micronuclei should be scored in at least 2 000 cells per concentration (at least 1 000 per culture, two cultures per concentration). Where only one cultures per concentration is used, at least 2 000 cells should be scored from that culture.

When cytoB is used, a CBPI or an RI should be determined to assess cell proliferation using at least 500 cells/culture.

8.5 Data and reporting

8.5.1 Treatment of results

If the cytokinesis-block technique is used, only the frequencies of binucleate cells with micronuclei are used in the evaluation of micronucleus induction. Scoring of the number of cells with one, two, or more micronuclei could provide useful information, but is not mandatory.

Concurrent measures of cytotoxicity and/or cytostasis for all treated and solvent/vehicle control cultures should be determined. The CBPI (Cytokinesis-Block Proliferation Index) or the RI (Replication Index) should be calculated for all treated and control cultures as measurements cell cycle delay when the cytokinesis-block method is used. In the absence of cytoB, the RPD (Relative Population Doubling) or the RICC (Relative Increase in Cell Count or PI (Proliferation Index) should be used.

Individual culture data should be provided and additionally, all data should be summarized in tabular form.

There is no requirement for verification of a clear positive or negative response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. The need to confirm negative results has been discussed previously. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

If, having selected a cell under low power, it is obvious under high power that the quality of the metaphase precludes accurate CA scoring (e.g. excessively “fuzzy” or overlapping chromosomes), it should not be included in the scoring. Initially, a minimum of 200 cells should be scored from each treatment group, 100 from each of two replicates. If ambiguous results are obtained, further “blind” scoring of these same samples may be undertaken including the additional replicates of negative or solvent controls.

8.5.2 Evaluation and interpretation of results

There is no requirement for verification of a clear positive or negative response. Equivocal results can be clarified by analysis of another 1 000 cells from all the cultures. If this approach does not resolve

the result, further testing should be performed. Modification of study parameters over an extended or narrowed range of conditions, as appropriate, should be considered in follow-up experiments.

There are several criteria for determining a positive result, such as a concentration-related increase or a statistically significant increase in the number of cells containing micronuclei. The biological relevance of the results should be considered first. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response.

Although most experiments will give clearly positive or negative results, the data set will preclude making a definite judgement about the activity of the test samples in rare cases. Results can remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* micronucleus assay indicate that the test sample induces chromosome breakage or loss, in cultured mammalian cells. Negative results indicate that, under the test conditions, the test sample does not induce chromosome breaks and loss in cultured mammalian somatic cells.

8.5.3 Test report

The test report should include the following information:

a) test sample:

   1) medical device, components, or materials tested;

   2) identification data and CAS no., if known;

   3) physical nature and purity of materials tested, if known;

   4) physicochemical properties relevant to the conduct of the study;

   5) stability of the test sample, if known;

   6) sterilization status;

   7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

   8) description of the test sample physical characteristics, e.g. clarity, colour, and presence of particulates;

   9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

b) solvent/vehicle:

   1) justification for choice of solvent/vehicle;

   2) solubility and stability of the test sample in solvent/vehicle, if known;

c) cells:

   1) type and source of cells;

   2) karyotype features and suitability of the cell type used;

   3) absence of mycoplasma, if applicable;

   4) information on cell cycle length;

   5) sex of blood donors, whole blood or separated lymphocytes, mitogen used;

   6) number of passages, if applicable;

7) methods for maintenance of cell cultures, if applicable;

8) modal number of chromosomes;

d) test conditions:

1) test sample preparation method with rationale for selection of method;

2) rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;

3) composition of media, CO_2 concentration, if applicable;

4) concentration of test sample or extract;

5) volume of vehicle and test sample or extract added;

6) incubation temperature;

7) incubation time;

8) duration of treatment;

9) cell density at seeding, if appropriate;

10) the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded. If the S9 is an in-house source, then source and method of preparation should be documented;

11) type and composition of metabolic activation system, including acceptability criteria;

12) positive and negative controls;

13) methods of slide preparation;

14) criteria for scoring micronuclei;

15) number of cells analysed;

16) methods for the measurements of toxicity;

17) criteria for considering studies as positive, negative or equivocal;

e) results:

1) signs of toxicity, e.g. CBPI, RI, RICC, RCC;

2) signs of precipitation;

3) data on pH and osmolality of the treatment medium, if determined;

4) number of cells with micronuclei for each treated and control culture;

5) dose-response relationship, where possible;

6) statistical analyses, if any;

7) concurrent negative (solvent/vehicle) and positive control data;

8) historical negative (solvent/vehicle) and positive control data, with ranges, means, and standard deviations;

f) discussion of the results;

g) conclusion.

9 *In vitro* mammalian cell gene mutation test using mouse lymphoma (L5178Y) cells

9.1 General

The following procedure for the *in vitro* mammalian cell gene mutation test using mouse lymphoma, L5178Y - Thymidine Kinase (TK)$^{+/-}$ cells was adopted for medical devices using OECD 476.[5] For evaluation of genotoxic potential of medical devices, medical device/material or extracts from medical device/material or extracted and evaporated residues from medical device/materials or chemicals can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Cells deficient in thymidine kinase (TK) due to the mutation TK$^{+/-}$ → TK$^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not.

9.2 Preparations

9.2.1 Cells

Subclones of mouse lymphoma cells, L5178Y TK$^{+/-}$, are used in this test. Sufficient quality control measures should be established to ensure identity and adequate performance of the cell lines with methods such as sensitivity to chemical mutagens, a high cloning efficiency, a stable spontaneous mutant frequency, or karyotyping.

Cells should be checked for mycoplasma contamination and should not be used if contaminated.

Include S9 concentration used in assay.

The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test sample or extract used should reflect these defined parameters.

The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency. A general guide is to use a cell number which is at least ten times the inverse of the spontaneous mutation frequency.

9.2.2 Media and culture conditions

Both RPMI-1640 and Fischer's Medium for Leukaemic Cells of Mice media have been successfully used with the MLA. It is particularly important that culture conditions should be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells.

The osmolalilty and pH of the medium should be in the physiological range.

9.2.3 Preparation of cultures

Cells are propagated from stock cultures, seeded in culture medium, and incubated at 37 °C. The cleansing procedure is conducted on two consecutive days. The cells are first treated with THMG (thymidine, hypoxanthine, methotrexate, glycine) solution weekly to cleanse the culture of spontaneous TK–/– mutants; followed by THG the following day.

9.2.4 Metabolic activation

Cells should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor supplemented post-mitochondrial

fraction S9 prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range of 1 % to 10 % volume fraction in the final test medium. The choice and condition of a metabolic activation system can depend upon the class of chemical being tested. In some cases, it can be appropriate to utilize more than one concentration of post-mitochondrial fraction.

The supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded, if the S9 is an in-house source, then source and method of preparation should be documented.

The buffer and component concentrations should be defined.

Simple omission of the S9 mix component is not recommended in the absence of metabolic activation system, as the differing volumes will alter the perceived dose of compound. S9 mix should be replaced with an appropriate buffer or culture medium.

9.2.5 Test sample preparations

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (see ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (see ISO 10993-3, Annex A, Method B and Method C).

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

9.3 Test conditions

9.3.1 Solvent/vehicle

The test solvents are selected in accordance with ISO 10993-12 or ISO 10993-3, Annex A and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvents are used, their inclusion should be supported by data indicating their compatibility.

9.3.2 Exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system, and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indicator of cell integrity and growth, such as relative cloning efficiency (survival) or relative total growth.[11] It can be useful to determine cytotoxicity and solubility in a preliminary experiment. For ISO 10993-12, a limit study using 100 % test sample extract is acceptable and no further dosing study is necessary. For ISO 10993-3, Annex A, the following should be considered.

The recommended maximum test concentration will depend on the test sample preparation method selected.

a) For soluble non-cytotoxic test samples (see ISO 10993-3, Annex A, Method A), the recommended maximum test concentration is 5 mg/ml or 5 µl/ml or 0,01 M. For non-cytotoxic test samples that are not soluble at 5 mg/ml or 5 µl/ml or 0,01 M, one or more concentrations tested should be insoluble in

the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml or 5 µl/ml or 0,01 M should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

b) For test samples prepared in accordance with ISO 10993-3, Annex A, Method B the recommended maximum test concentration is 5 mg/ml if feasible. For non-cytotoxic test samples that are not soluble at 5 mg/ml or 5 µl/ml or 0,01 M, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/ml or 5 µl/ml or 0,01 M should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

c) For test samples prepared in accordance with ISO 10993-3, Annex A, Method C the recommended maximum test concentration is 100 % of the test sample extract. For non-cytotoxic test samples that are not soluble at 100 %, one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 100 % should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

A single top dose is adequate for non-cytotoxic, soluble residues. At least four analysable concentrations should be used when cytotoxicity is observed. These concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentration levels should be separated by no more than a factor between 2 and the square root of 10. If the maximum concentration is based on cytotoxicity, then it should result in approximately 10 % to 20 % (but not less than 10 %) relative survival (relative cloning efficiency) or relative total growth.

For relatively non-cytotoxic test samples, the maximum concentration should be 5 µl/ml, 5 mg/ml or 0.01 M, whichever is the lowest. Under this circumstance, a single maximum concentration is acceptable.

For relatively insoluble test samples or extracts that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration), it is advisable to test at more than one concentration with visible precipitation. It can be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum, etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

9.3.3 Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be one that requires activation to give a mutagenic response.

Examples of positive control substances include the following:

a) absence of exogenous metabolic activation:

   1) Methylmethanesulfonate [CAS no. 66-27-3];

b) presence of exogenous metabolic activation:

   1) Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (6055-19-2)];

   2) Benzo(a)pyrene [CAS no. 50-32-8] 3;

   3) Methylcholanthrene [CAS no. 56-49-5].

Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals can be considered, when available.

Positive control responses with and without S9 should be used for quality control measures and to demonstrate adequate detection of small colony mutants. Each laboratory should establish its own historical database for its positive and negative controls.

Negative controls consisting of solvent or vehicle alone in the treatment medium and treated in the same way as the treatment groups should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

9.4 Procedure

9.4.1 General

Cytotoxicity should be determined for each individual test and control culture. For the soft agar version of the MLA, this has generally been done using the relative total growth (RTG) which was originally defined by Reference [11]. This measure includes the relative growth in suspension during the expression time and the relative cloning efficiency at the time that mutants are selected. The microwell version of the assay was developed using the relative survival (RS) as the cytotoxicity measure. The RS is determined by the relative plating efficiency of each culture when plated immediately after the exposure period. The RTG and the RS are different measures of cytotoxicity and, although there is no real justification that one measure is superior to the other, it is important that the same measure of cytotoxicity be used for both versions of the assay. Because the RS is not normally measured in the soft agar version of the assay and the RTG is measured in both versions, it is recommended that the RTG be used as the standard measure of cytotoxicity. This cytotoxicity value is used both to determine the required concentration range for an acceptable test and for establishing the highest concentration that is used for defining positive and negative responses.

There are additional considerations in the calculation of the RTG between the two methods for the conduct of the MLA. In the agar method, the cells are exposed to the test chemicals; the chemical is removed by centrifugation and re-suspension in fresh medium. The first cell count takes place approximately 24 h after the initiation of the chemical exposure. On the first day following treatment, the cell density for each culture is readjusted, generally to 0,2 or 0,3 × 10^6 cell/ml of medium. Treated cultures with densities less than 0,2 or 0,3 × 10^6 cell/ml of medium are generally not adjusted in their density and usually have sustained too much cytotoxicity to carry through the full experiment for mutant enumeration. For each treatment culture, the relative cell growth (compared to control) is calculated. On the second day following treatment, the cultures are again counted, adjusted in density and prepared to clone for mutant enumeration. The total two-day suspension growth of each culture is calculated and each treated culture is compared to the control. This value is referred to as the relative suspension growth (RSG). Cultures are cloned with and without selective medium to enumerate mutants and to calculate the mutant frequency (number of mutants per 10^6 cloneable cells). The relative plating efficiency for each culture is determined (relative to the negative control) and multiplied by the RSG to obtain a relative total growth (RTG).

In the microwell method, most laboratories count the cell cultures immediately following exposure to the test chemical and adjust the density of the cultures. Following the end of treatment and the adjustment of cell density, the cell cultures are handled just like the cultures in the agar method. Following the two-day expression period, the cultures are plated in 96-well plates, with and without TFT selection.

As described above, handling of the cell cultures following treatment differs significantly between the two methods. This difference impacts the calculation of the RSG and RTG. The RSG and the RTG, in the agar method, are calculated to include any differences that can occur in cell growth between the chemically treated and control cultures. However, in the microwell method, the cultures are generally adjusted in density following treatment and the RS, RSG, and RTG calculated using the plating efficiency and cell growth that occurs following treatment. In other words, any differential growth that occurs between the negative controls and the treatment cultures during the treatment phase of the assay is not factored into the calculation.

To make the cytotoxicity measures obtained in the two versions equivalent, it is necessary for users of the microwell method to adjust their RS, RSG, and RTG values to include the differential growth that can occur during treatment. This adjustment should be made by comparing the cell density in each treated culture with that of the negative control immediately following treatment. By comparing the growth of each treated culture relative to the control, it is possible to calculate a relative growth during treatment factor that can then be used to adjust the RS, RSG, and RTG. As an example, if following the treatment

period, the negative control had a cell density of 0,6 × 10^6 cell/ml and the treated culture had a density of 0,3 × 10^6 cell/ml, then the relative growth during treatment for that treated culture is 0,5 (or 50 %). If the RS for that culture is determined to be 0,4, then the adjusted RS would be calculated as the RS × the relative growth during treatment or 0,4 × 0,5 = 0,20 (or 20 %). The RSG would be adjusted in the same manner. The adjusted RTG would be obtained by multiplying the adjusted RSG by the relative plating efficiency at the time of mutant selection.

9.4.2 Treatment with test sample

Proliferating cells should be exposed to the test sample both with and without metabolic activation. Exposure should be for a suitable period of time (usually 3 h to 4 h is effective). If the conclusion is negative following the 3 h to 4 h treatment, evaluate the test sample (without metabolic activation) using a 24 h treatment period. The 24 h treatment may also be performed concurrent to the short time point.

Either duplicate or single treated cultures may be used at each concentration tested. When single cultures are used, the number of concentrations should be increased to ensure an adequate number of cultures for analysis (e.g. at least eight analysable concentrations). Duplicate negative (solvent) control cultures should be used.

9.4.3 Measurement of survival, viability and mutant frequency

At the end of the exposure period, cells are washed and cultured to determine survival and to allow for expression of the mutant phenotype. Measurement of cytotoxicity by determining the relative cloning efficiency (survival) or relative total growth of the cultures is usually initiated after the treatment period.

The cells are cultured for at least two additional days to allow near optimal phenotypic expression of newly induced mutants for thymidine kinase. The cells are then grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively. The measurement of viability (used to calculate mutant frequency) is initiated at the end of the expression time by plating in non-selective medium.

This mutant selection can be accomplished using TFT selection, see Reference [22], and either the soft agar or the microwell cloning method, see Reference [23].

In the soft agar method, the mutant frequency (MF) is determined by counting the number of TFT resistant colonies and correcting the number of cells plated for selection by the plating efficiency (PE). That is, the MF = (number of mutants/number of cells plated) × PE. For the microwell method, the plating efficiency (PE) and the mutant frequency (MF) are calculated using the Poisson distribution. The plating efficiency (PE) in both the mutant selection plates and the viability plates is calculated as follows: From the zero term of the Poisson distribution, the probable number of clones/well (P) is equal to -ln(EW/TW), where EW = empty wells and TW = total wells. The PE = P/Number of cells plated per well. The mutant frequency is then calculated: MF = [PE(mutant)/PE(viable)] × 10^6.

If the test substance is negative, mutant colony sizing should be performed on the negative and positive controls. Colony sizing on the negative control is needed to demonstrate that large colonies are growing adequately. The test chemical cannot be determined to be negative if the positive control does not demonstrate the appropriate level of small mutant colony induction and detection.

9.5 Data and reporting

9.5.1 Treatment of results

Data should include cytotoxicity and viability determination, colony counts, and mutant frequencies for the treated and control cultures. In the case of a positive response in the L5178Y TK+/- test, colonies are scored using the criteria of small and large colonies on at least one concentration of the test sample (highest positive concentration) and on the negative and positive control. The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail. In the TK+/- test, colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies. Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus, form small

colonies. This damage typically ranges in scale from the losses of the entire gene to karyotypically visible chromosome aberrations. The induction of small colony mutants has been associated with chemicals that induce gross chromosome aberrations. Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

Survival (relative cloning efficiencies) or relative total growth should be given. Mutant frequency should be expressed as number of mutant cells per number of surviving cells.

Individual culture data should be provided. Additionally, all data should be summarized in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

9.5.2 Evaluation and interpretation of results

9.5.2.1 General

There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response.

A test sample or extract, for which the results do not meet the above criteria, is considered nonmutagenic in this system.

Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test sample. Results can remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results for an *in vitro* mammalian cell gene mutation test indicate that the test sample induces gene mutations in the cultured mammalian cells used. A positive concentration response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test sample does not induce gene mutations in the cultured mammalian cells used.

9.5.2.2 Criteria for the determination of a valid test

The following criteria should be met for the mutagenesis assay to be considered valid:

Negative Controls: The average spontaneous mutant frequency of the solvent (or vehicle) control cultures should be within 35 to 140 TFT-resistant mutants per 10^6 surviving cells. Low spontaneous mutant frequencies, i.e. 20 to 34 mutants per 10^6 surviving cells, are considered acceptable if small colony recovery is demonstrated, see Reference [19]. The average cloning efficiency of the solvent (or vehicle) controls should be between 60 % and 120 % and the total suspension growth between 8 and 32 for the 4 h exposure (see Reference [20] and Reference [21]).

Positive Controls: The mutant frequency for at least one dose of the positive controls should meet the criteria for a positive response and induce an increase in small colony mutants in accordance with the following criteria

— 4 h: ≥100 mutants over the negative control, or

— 24 h – as defined by Induced Mutant Frequency (IMF) positive control ≥ 300 × 10-6 mutants with 40 % small colonies, or

— small colony IMF for positive control ≥150 × 10^{-6}, (see Reference [20] and Reference [21])

9.5.2.3 Test report

The test report should include the following information:

a) test sample or extract:

1) medical device, components, or materials tested;

2) identification data and CAS no., if known;

3) physical nature and purity of materials tested, if known;

4) physicochemical properties relevant to the conduct of the study;

5) stability of the test sample, if known;

6) sterilization status;

7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

8) description of the test sample physical characteristics, e.g. clarity, colour, and presence of particulates;

9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

10) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

b) solvent/vehicle:

1) justification for choice of solvent/vehicle;

2) solubility and stability of the test sample in solvent/vehicle, if known;

c) cells:

1) type and source of cells;

2) number of cell cultures;

3) number of cell passages, if applicable;

4) methods for maintenance of cell cultures, if applicable;

5) absence of mycoplasma;

d) test conditions:

1) rationale for selection of concentrations and number of cell cultures including e.g. cytotoxicity data and solubility limitations, if available;

2) test sample preparation method with rationale for selection of method;

3) composition of media, CO_2 concentration;

4) concentration of test sample or extract;

5) volume of vehicle and test sample or extract added;

6) incubation temperature;

7) incubation time;

8) duration of treatment;

9) cell density during treatment;

10) the supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded. If the S9 is an in-house source, then source and method of preparation should be documented. S9 concentration used in assay should be included;

11) type and composition of metabolic activation system including acceptability criteria;

12) positive and negative controls;

13) length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);

14) selective agent(s);

15) criteria for considering tests as positive, negative or equivocal;

16) methods used to enumerate numbers of viable and mutant cells;

17) definition of colonies of which size and type are considered (including criteria for "small" and "large" colonies, as appropriate);

e) results:

1) signs of toxicity;

2) signs of precipitation;

3) data on pH and osmolality during the exposure to the test sample, if determined;

4) colony size if scored for at least negative and positive controls;

5) laboratory's adequacy to detect small colony mutants with the L5178Y TK^+/system, where appropriate;

6) dose-response relationship, where possible;

7) statistical analyses, if any;

8) concurrent negative (solvent/vehicle) and positive control data;

9) historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations;

10) mutant frequency;

f) discussion of the results;

g) conclusion.

10 *In vivo* mammalian erythrocyte micronucleus test

10.1 General

The following procedure for the *in vivo* Mammalian Erythrocyte Micronucleus Test was adopted for medical devices using OECD 474.[3] For evaluation of genotoxic potential of medical devices, medical device/material or extracts from medical device/material or extracted and evaporated residues from medical device/materials or chemicals can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Animals are exposed to the test sample by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and smears (slides) are prepared and stained. When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained. For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analysed for the presence of micronuclei.

10.2 Preparations

10.2.1 Selection of animal species

Mice or rats are recommended if bone marrow is used, although any other appropriate mammalian species may be used. When peripheral blood is used, mice are recommended.

However, any other mammalian species may be used in which the spleen does not remove micronucleated polychromatic erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numeral chromosome aberrations or induce damage to the chromosome or the mitotic apparatus of polychromatic erythrocytes. Commonly used laboratory strains of young healthy animals (6 weeks to 12 weeks) should be employed to avoid fatty deposition in the marrow which can reduce the clarity of stained preparations. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 % of the mean weight of each sex.

10.2.2 Housing and feeding conditions

The temperature in the experimental animal room should be (22 ± 3) °C. Animals may be housed individually or caged in small groups (up to six) of the same sex. Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50 % to 60 %. Lighting should be artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet can be influenced by the need to ensure a suitable admixture of a test sample or extract when administered by this route.

10.2.3 Preparation of the animals

Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized.

10.2.4 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (see ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (see ISO 10993-3, Annex A, Method B and Method C) or the outcome of *in vitro* tests.

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

10.3 Test conditions

10.3.1 Solvent/vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reaction with the test sample. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility.

Test samples should be prepared in accordance with ISO 10993-12 or ISO 10993-3, Annex A.

10.3.2 Controls

Concurrent positive and negative (vehicle) controls should be included for each sex. Except for the treatment with the test sample preparation, animals in the control groups should be handled in an identical manner to animals of the test sample preparation treatment groups.

Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test sample preparation and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include the following:

— Ethyl methanesulphonate [CAS no. 62-50-0];

— Ethyl nitrosourea [CAS no. 759-73-9];

— Mitomycin C [CAS no. 50-07-7];

— Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)];

— Triethylenemelamine [CAS no. 51-18-3];

— MMS.

Negative control animals are treated with solvent or vehicle alone and otherwise handled in the same way as the animals in the test sample treatment groups. If acceptable interanimal variability and frequencies of cells with micronuclei are demonstrated by historical control data, only one negative control group at the first sample time might be needed.

If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short-term studies [e.g. one to three treatment(s)] if the resulting data are in the expected range for the historical control.

10.4 Procedure

10.4.1 Number and sex of animals

Each treated and control group should include at least five analysable animals per sex. If, at the time of the study, there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals might be sex specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

10.4.2 Treatment schedule

The test may be performed in mice or rats in three ways as follows.

a) Animals are treated with the test substance once or test substances also may be administered as a split dose. Samples of bone marrow are taken at least twice, starting not earlier than 24 h after

treatment, but not extending beyond 48 h after treatment with appropriate interval(s) between samples, unless the test substance is known to have an exceptionally long half-life. The use of sampling times earlier than 24 h after treatment should be justified. Samples of peripheral blood are taken at least twice, starting not earlier than 36 h after treatment, with appropriate intervals following the first sample, but not extending beyond 72 h. When a positive response is detected at one sampling time, additional sampling is not required unless quantitative dose-response information is needed.

b) If two daily treatments are used (e.g. two treatments at 24 h intervals), samples should be collected once between 18 h and 24 h following the final treatment for the bone marrow and once between 24 h and 48 h following the final treatment for peripheral blood.

c) If three or more daily treatments are used (e.g. three or more treatments at 24 h intervals), bone marrow samples should be collected no later than 24 h after the last treatment. Peripheral blood should be collected no later than 40 h after the last treatment; however, approximately 24 h after the last treatment is most practical.

Other sampling times may be used when relevant and justified scientifically.

Increased dose studies may be preferred if the test chemical is posing formulation problems, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. In this way, a higher total dose can be given. The choice of procedure should be made on the basis of the knowledge of the toxicity data, if available.

The samples from extended dose regimens (e.g. 28-day daily dosing) are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity to the erythropoietic system has been demonstrated or the limit dose has been used and dosing continued until the time of sampling.

10.4.3 Limit test

For studies based on doses at a specific mg/kg body weight, a full study using three dose levels might not be considered necessary if

— dose range-finding experiments or existing data from related animal strains indicate that a treatment regime of at least the limit dose (described below) produces no observable toxic effects (including no depression of bone marrow proliferation), and

— genotoxicity would not be expected based upon *in vitro* genotoxicity studies or data from structurally related substances.

In such cases, a single dose level, at the limit dose, may be sufficient. For an administration period of 14 days or more, the limit dose is 1 000 mg/kg body weight/day. For administration periods of less than 14 days, the limit dose is 2 000 mg/kg/body weight/day.

For studies using ISO 10993-12 extracts, a study using 100 % test sample extract is acceptable and no further dosing study is necessary.

Expected human exposure can indicate the need for a higher dose level to be used in the limit test.

10.4.4 Dose levels

In cases where toxicity is expected, a dose range finding (DRF) study may be conducted. This study may be conducted prior to the main study in order to determine the toxicity of the test sample preparation and set dose levels for the main study.

In the main study, the dose levels for bone marrow/blood analysis should not be doses with mortality but should cover a range from the maximum to little or no toxicity. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. The testing should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the

dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity of the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

10.4.5 Routes of administration and doses levels

Saline solutions can be injected intravenously at up to 20 ml/kg for mice and up to 10 ml/kg for rats; organic vehicles or extracts may be injected intraperitoneally at 20 ml/kg for mice and 10 ml/kg for rats. Other routes of exposure may be acceptable where they can be justified and the extract is shown to be taken up by this route. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. Except for irritating or corrosive substances which will normally induce exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels. The use of volumes different than those mentioned above should be justified.

10.4.6 Bone marrow/blood preparation

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Commonly, cells are removed from femurs, tibias, or humerus bone, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally or smear preparations are made and then stained. Alternatively, for samples analysed using flow cytometry, specimens are prepared using appropriate, validated methods. The use of a DNA specific stain (e.g. acridine orange or Hoechst 33258[3)] plus pyronin-Y) can eliminate some of the artefacts associated with using a non-DNA specific stain. This advantage does not preclude the use of conventional stains (e.g. Giemsa). Additional methods (e.g. cellulose columns to remove nucleated cells) can also be used provided that these methods have been shown to adequately work for micronucleus preparation in the laboratory.

10.4.7 Analysis

Microscopic evaluation of the bone marrow or peripheral blood is the accepted scoring method, however, systems for automated analysis (image analysis and cell suspensions flow cytometry) are an acceptable alternative to manual evaluation if appropriately justified and validated.

To reduce potential observer bias in microscopic evaluation, all slides, including those of positive and negative controls, should be randomized and coded (to reduce the likelihood of identifying the sample) before microscopic analysis by one scorer. It is essential that the micronuclei be defined prior to scoring.

For the bone marrow and peripheral blood, using microscopic method, at least 2 000 polychromatic erythrocytes (PCEs)/reticulocytes (RET) per animal are scored for the incidence of micronucleated polychromatic erythrocytes (MN-PCE)/reticulocytes (MN-RET).

For the peripheral blood, using cytometric Method Approximately 20 000 reticulocytes (young erythrocytes) per animal are scored. Additional information can be obtained by scoring normochromatic erythrocytes (NCEs) for the presence of micronuclei (MN-NCEs).

In order to quantify the test compounds/test substance extracts effect on erythropoiesis as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes (PCEs/ECs ratio where ECs = PCEs + MN-PCEs + NCEs + MN-NCEs)) is determined per a total of at least 200 erythrocytes for each animal. The proportion of polychromatic erythrocytes to total erythrocytes in test compound/test substance extract-treated animals should not be reduced below 80 % of the negative control value.

The percentage of reticulocytes in peripheral blood will be calculated per total number of erythrocytes enumerated for each animal and treatment group. The percentage of reticulocytes in the test

3) Hoechst 33258 can be obtained from Hoechst GmbH, D-65926 Frankfurt am Main. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

compound/test substance extract groups should be comparable with the negative control groups otherwise the percentage would indicate toxicity.

10.5 Data and reporting

10.5.1 Evaluation of results

Individual bone marrow animal data should be presented in tabular form. The experimental unit is the animal. For each male and female animal, the following data should be recorded: the number of polychromatic erythrocytes, the number of normochromatic erythrocytes, and the number of micronucleated polychromatic erythrocytes and micronucleated normochromatic erythrocytes. The ratio of polychromatic erythrocytes to total erythrocytes (PCEs/ECs ratio) should be calculated for each animal. Summary tables should show the incidence of micronucleated polychromatic erythrocytes per group, mean incidence of micronucleated polychromatic erythrocytes, and the ratio of polychromatic erythrocytes to total erythrocytes at each sampling time for each treatment group.

In case of an increased frequency of micronucleated polychromatic erythrocytes in the test sample, micronucleated normochromoatic erythrocytes may be scored for quality control purposes since artefacts in any given slide will produce apparent increases in "micronucleus" incidence in both normochromatic and polychromatic erythrocytes. The mean incidence of micronucleated normochromatic erythrocytes may also be tabulated.

If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

Some other alternatives in results presentation may be acceptable.

10.5.2 Evaluation and interpretation of results

Most experiments will give clearly positive or negative results based on statistical analysis. However, the results of a statistical analysis might not be the only criteria in evaluation of the test sample positivity. In interpreting the data, the following may be taken in consideration: results, in rare cases the data set will preclude making a definite judgement about the activity of the test sample. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Negative results indicate that, under the test conditions, the test sample does not produce micronuclei in the immature erythrocytes of the test species.

The likelihood that the test sample, extract, or its metabolites reach the general circulation or specifically, the target tissue (e.g. systemic toxicity) should be discussed.

The number of mean micronucleated polychromatic erythrocytes in the vehicle (negative) control group should not exceed 4 PCE per 1 000 cells

If criteria for either a positive or negative genotoxic response are not met, the results may be judged as equivocal or inconclusive and further testing preferably using a modification of experimental conditions should be conducted. Additional PCE (+2 000) may be scored from each treated and control animal to confirm the result. If the response is real and not due to sampling error, statistical significance should increase with increased sample size.

10.5.3 Test report

The test report should also include the following information:

a) test sample or extract:

   1) medical device, components or materials tested;

   2) identification data and CAS no., if known;

3) physical nature and purity of material tested, if known;

4) physicochemical properties relevant to the conduct of the study;

5) stability of the test sample, if known;

6) sterilization status;

7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

8) description of the test sample physical characteristics, e.g. clarity, colour, and presence of particulates;

9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

10) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

b) solvent/vehicle:

1) justification for choice of vehicle;

2) solubility and stability of the test sample in the solvent/vehicle, if known;

c) test animals:

1) species/strain used;

2) number, age and sex of animals;

3) source, housing conditions, diet, etc;

d) results:

1) signs of toxicity;

2) sampling time for each treatment group;

3) proportion of immature erythrocytes among total erythrocytes;

4) number of micronucleated immature erythrocytes, given separately for each animal;

5) mean standard deviation of micronucleated immature erythrocytes per group;

6) dose-response relationship, where possible;

7) statistical analyses and Method A applied;

8) concurrent and historical negative control data;

9) concurrent positive control data;

10) individual body weights and group mean weights (per sex);

11) individual animal clinical observations;

e) discussion of the results;

f) conclusion.

11 Chromosome aberration test (*in vivo*)

11.1 General

The following procedure for the *in vivo* Chromosome aberration test was adopted for medical devices using OECD 475.[4] For evaluation of genotoxic potential of medical devices, medical device/material, or extracts from medical device/material or extracted and evaporated residues from medical device/materials or chemicals can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Animals are exposed to the test sample by an appropriate route. The animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained. Preparations are examined and metaphase cells are scored for structural chromosomal aberrations.

11.2 Preparations

11.2.1 Selection of animal species

Mice at the age of 6 weeks to 12 weeks or rats at the age of 8 weeks to 12 weeks are recommended, although any appropriate mammalian species may be used. Commonly used laboratory strains of young, healthy animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 % of the mean weight of each sex.

11.2.2 Housing and feeding conditions

The temperature in the experimental animal room should be 18 °C to 26 °C. Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50 % to 60 %. Lighting should be artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test sample or extract when administered by this route. Animals may be housed individually or caged in small groups of the same sex.

11.2.3 Preparation of the animals

Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized.

11.2.4 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (see ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (see ISO 10993-3, Annex A, Method B and Method C) or the outcome of *in vitro* tests.

Test extracts should be used within 24 h of preparation. If test extracts are stored up to 24 h, the storage condition should be stated.

11.3 Test conditions

11.3.1 Solvent/vehicle

The solvent/vehicle should be selected in accordance with ISO 10993-12 or ISO 10993-3, Annex A and should not produce toxic effects at the dose levels used. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility.

11.3.2 Controls

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test sample, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

Positive controls should induce structural chromosomal aberrations *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test sample and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include the following:

— Ethyl methanesulphonate [CAS no. 62-50-0];

— Ethyl nitrosourea [CAS no. 759-73-9];

— Mitomycin C [CAS no. 50-07-7];

— Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)];

— Triethylenemelamine [CAS no. 51-18-3].

Negative controls treated with solvent or vehicle alone and otherwise treated in the same way as the treatment groups should be included for every sampling time, unless acceptable interanimal variability and frequencies of cells with structural chromosomal aberrations are demonstrated by historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time.

11.4 Procedure

11.4.1 Number and sex of animals

Each treated and control group should include at least five analysable animals per sex. If, at the time of the study, there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals can be sex specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

11.4.2 Treatment schedule

Approximately 18 h before treatment with the test item, the animals receive no food but water ad libitum. At the beginning of the treatment, the animals are weighed and the individual volume to be administered is adjusted to the animal's body weight. The animals will receive the test item once. Twelve animals (six males and six females) will be treated per dose group. The animals will be examined for acute toxic symptoms around 1 h, 2 h to 4 h, 6 h und 24 h after treatment.

Prior (2,5 h) to sacrifice, animals are injected intraperitoneally with the spindle inhibitor Colcemid®[4] (2,0 mg/kg body weight), to arrest cells in metaphase.

11.4.3 Dose levels

In cases where toxicity is expected, a dose range finding (DRF) study may be conducted. This study may be conducted prior to the main study in order to determine the toxicity of the test sample preparation and set dose levels for the main study.

If a range finding study is performed, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study. If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time, only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) can be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity of the bone marrow (e.g. reduction of the mitotic index).

11.4.4 Limit test

For studies based on doses at a specific mg/kg body weight, a full study using three dose levels might not be considered necessary if

— dose range-finding experiments, or existing data from related animal strains, indicate that a treatment regime of at least the limit dose (described below) produces no observable toxic effects (including no depression of bone marrow proliferation), and

— genotoxicity would not be expected based upon *in vitro* genotoxicity studies or data from structurally related substances

In such cases, a single dose level, at the limit dose, may be sufficient. For an administration period of 14 days or more, the limit dose is 1 000 mg/kg body weight/day. For administration periods of less than 14 days, the limit dose is 2 000 mg/kg/body weight/day.

For studies using ISO 10993-12 extracts, a study using 100 % test sample extract is acceptable and no further dosing study is necessary.

Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

11.4.5 Dose levels and routes of exposure

Saline solutions can be injected intravenously at up to 20 ml/kg for mice and up to 10 ml/kg for rats; organic vehicles or extracts may be injected intraperitoneally at 20 ml/kg for mice and 10 ml/kg for rats. Other routes of exposure may be acceptable where they can be justified and the extract is shown to be taken up by this route. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels. The use of volumes different than those mentioned above should be justified.

In order to arrest the cells in metaphase, all animals will receive a single intraperitoneal injection (IP) of colchicine at a dose level of approximately 2 mg/kg body weight. Colchicine will be administered to the animals at 2 h to 4 h prior to scheduled bone marrow collection time.

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4) Colcemid is the trade name of a product supplied by Ciba-Geigy Company, Basel, Switzerland. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

11.4.6 Bone marrow collection and preparation of slides

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. In order to arrest the cells in metaphase, the bone marrow cell suspension will be exposed to a hypotonic solution of KCl, fixed and dropped on slides. The metaphase spreads are stained with Giemsa.

11.4.7 Analysis of Metaphase Cells

Evaluation of the slides is performed microscopically and structural and numerical aberrations are recorded. At least 100 well spread metaphases per animal are scored for chromosome damage on coded slides. Structural aberration evaluations should record the following:

— chromatid-type aberrations (chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements;

— chromosome-type aberrations: chromosome breaks and exchange figures such as dicentric chromosomes and rings;

— fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange;

— pulverized chromosome(s), pulverized cells, and severely damaged cells (≥10 aberrations) will also be recorded;

— chromatid and isochromatid gaps will be recorded but not included in the analysis chromatid-type aberrations.

The numerical aberration (percent polypoloid and endoreduplicated cells) will be evaluated per 100 metaphase cells per animal. The Mitotic Index (MI) will be recorded as the percentage of cells in mitosis based upon 1 000 cells counted per animal. The mean MI will be calculated for each treatment group (including positive and negative control groups) and will serve as a parameter for inhibition of cell division and cytotoxicity.

11.5 Data and reporting

11.5.1 Treatment of results

The experimental unit is the animal. Individual animal data should be presented in tabular form along with means, standard deviation, and statistical significance, if any. The number of cells with chromosomal aberrations and the mitotic index should be listed separately for each animal analysed.

11.5.2 Evaluation and interpretation of results

There are several criteria for determining a positive response, such as a clear increase in the number of cells with structural chromosomal aberrations in a single dose group at a single sampling time or a dose-related increase in the number of cells with structural chromosomal (when a dose range study is needed for toxic residues). Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

If the test material has been administered at the highest dose that can be formulated and administered or at an upper limit of 2 000 mg/kg and a significant increase in cells with structural chromosomal aberration is not observed, an agent should be classified as negative under the conditions of the test.

A test sample or extract for which the results do not meet the above criteria is considered non-clastogenic in this test.

Although most experiments will give clearly positive or negative results, the data set will preclude making a definite judgement about the activity of the test sample in rare case. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results in the chromosome aberration test indicate that a substance induces structural chromosomal aberrations which are the result of chromosomal damage in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test sample does not produce structural chromosomal aberrations.

The likelihood that the test sample, extract, or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

The aberration frequency of the vehicle control is below 2 %.

The incidence of structural chromosomal aberrations in the positive control group should be significantly increased relative to the vehicle (negative) control.

11.5.3 Test report

The test report should also include the following information:

a) test sample or extract:

   1) medical device, components or materials tested;

   2) identification data and CAS no., if known;

   3) physical nature and purity of material tested, if known;

   4) physicochemical properties relevant to the conduct of the study;

   5) stability of the test sample, if known;

   6) sterilization status;

   7) description of test sample preparation, to include rationale for selection of method, solvent, and conditions of sample preparation;

   8) description of the test sample physical characteristics, e.g. clarity, colour, and presence of particulates;

   9) information on duration of extraction as measured and elapsed time between sample preparation and use in the test;

   10) test sample preparation method with rationale for selection of the method used. For ISO 10993-3, Method B, the % residual determined in the pilot extraction study should be included;

b) solvent/vehicle:

   1) justificationfor choice of vehicle;

   2) solubility and stability of the test sample in the solvent/vehicle, if known;

c) test animals:

   1) species/strain used;

   2) number, age, and sex of animals;

   3) source, housing conditions, diet, etc.;

   4) individual weight of the animals at the start and end of the test, including body weight range, mean, and standard deviation for each group;

5) individual clinical observations of the animals should be recorded;

d) test conditions:

1) positive and negative (vehicle/solvent) control data;

2) test sample preparation method with rationale for selection of method;

3) data from range-finding study, if conducted;

4) rationale for dose level selection;

5) details of test sample or extract preparation;

6) details of the administration of the test sample;

7) rationale for route of administration;

8) methods for verifying that the test sample reached the general circulation or target tissue, if applicable;

9) conversion from diet/drinking water test sample or extract concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;

10) details of food and water quality;

11) detailed description of treatment and sampling schedules;

12) methods of slide preparation;

13) methods for measurement of toxicity;

14) criteria for scoring structural chromosomal aberrations in the bone marrow cells;

15) number of cells analysed per animal;

16) criteria for considering studies as positive, negative or equivocal;

e) results:

1) signs of toxicity;

2) cells in mitosis – mitotic index determination;

3) number of cells with structural and numerical chromosomal aberrations, given separately for each animal;

4) dose-response relationship, where possible;

5) statistical analyses and Method Applied;

6) concurrent and historical negative control data;

7) concurrent positive control data;

f) discussion of the results;

g) conclusion.

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General

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[2] OECD. 473, *In vitro Mammalian Chromosome Aberration Test*

[3] OECD. 474, *Mammalian Erythrocyte Micronucleus Test*

[4] OECD. 475, *Mammalian Bone Marrow Chromosome Aberration Test*

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