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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed and revised in the light of scientific progress, regulatory needs and animal welfare. The mouse lymphoma assay (MLA) and TK6 test using the thymidine kinase (TK) locus were originally contained in Test Guideline 476 adopted in 1984 and revised in 1997, based on scientific progress made to that date. Subsequently, the MLA Expert Workgroup of the International Workshop for Genotoxicity Testing (IWGT) has developed internationally harmonized recommendations for assay acceptance criteria and data interpretation for the MLA (1) (2) (3) (4) (5), and these recommendations are incorporated into this new Test Guideline (TG 490). TG 490 is written for the MLA and, because it also utilizes the TK locus, the TK6. While the MLA has been widely used for regulatory purposes, the TK6 has been used much less frequently. It should be noted that in spite of the similarity between the endpoints the two cell lines are not interchangeable and regulatory programs may validly express a preference for one over the other for a particular regulatory use. For instance, the validation of the MLA demonstrated its appropriateness for detecting not only gene mutation, but also, the ability of a test chemical to induce structural chromosomal damage. This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (6).

2. The purpose of the in vitro mammalian cell gene mutation tests is to detect gene mutations induced by chemicals. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogeneous thymidine kinase gene (*TK* for human cells and *Tk* for rodent cells, collectively referred to as *TK* in this Guideline). This Guideline is intended for use with two cell lines: the L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma cell line (generally called L5178Y) and the TK6 human lymphoblastoid cell line (generally called TK6). Although the two cell lines vary because of their origin, cell growth, p53-status, etc., the *TK* gene mutation tests can be conducted in a similar way in both cell types as described in this guideline.

3. The autosomal and heterozygous nature of the thymidine kinase gene enables the detection of viable colonies whose cells are deficient in the enzyme thymidine kinase following mutation from $TK^{+/-}$ to $TK^{-/-}$. This deficiency can result from genetic events affecting the *TK* gene including both gene mutations (point mutations, frame-shift mutations, small deletions, etc.) and chromosomal events (large deletions, chromosome rearrangements and mitotic recombination). The latter events are expressed as loss of heterozygosity, which is a common genetic change of tumor suppressor genes in human tumorigenesis. Theoretically, loss of the entire chromosome carrying the *TK* gene resulting from spindle impairment

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and/or mitotic non-disjunction can be detected in the MLA. Indeed, a combination of cytogenetic and molecular analysis clearly shows that some MLA TK mutants are the result of nondisjunction. However, the weight of evidence shows that the *TK* gene mutation tests cannot reliably detect aneugens when applying standard cytotoxicity criteria (as described in this guideline) and therefore, it is not appropriate to use these tests to detect aneugens (7) (8) (9).

4. In the *TK* gene mutation tests, two distinct phenotypic classes of *TK* mutants are generated; the normal growing mutants that grow at the same rate as the TK heterozygous cells, and slow growing mutants which grow with prolonged doubling times. The normal growing and slow growing mutants are recognized as large colony and small colony mutants in the MLA and as early appearing colony and late appearing colony mutants in the TK6. The molecular and cytogenetic nature of both large and small colony MLA mutants has been explored in detail (8) (10) (11) (12) (13). The molecular and cytogenetic nature of the early appearing and late appearing TK6 mutants has also been extensively investigated (14) (15) (16) (17). Slow growing mutants for both cell types have suffered genetic damage that involves putative growth regulating gene(s) near the *TK* locus which results in prolonged doubling times and the formation of late appearing or small colonies (18). The induction of slow growing mutants has been associated with substances that induce gross structural changes at the chromosomal level. Cells whose damage does not involve the putative growth regulating gene(s) near the TK locus grow at rates similar to the parental cells and become normal growing mutants. The induction of primarily normal growing mutants is associated with substances primarily acting as point mutagens. Consequently it is essential to count both slow growing and normal growing mutants in order to recover all of the mutants and to provide some insight into the type(s) of damage (mutagens vs. clastogens) induced by the test chemical (10) (12) (18) (19).

5. The Test Guideline is organized so as to provide general information that applies to both MLA and TK6 and specialised guidance for the individual tests.

6. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.

8. Care should be taken to avoid conditions that could lead to artifactual positive results (i.e. possible interaction with the test system) not caused by interaction between the test chemical and the genetic material of the cell; such conditions include changes in pH or osmolality, interaction with the medium components (20) (21), or excessive levels of cytotoxicity (22) (23) (24). Cytotoxicity exceeding the recommended top cytotoxicity levels as defined in paragraph 28 is considered excessive for the MLA and TK6. In addition, it should be noted that test chemicals that are thymidine analogues, or behave like thymidine analogues can increase the mutant frequency by selective growth of the spontaneous background mutants during cell treatment and require additional test methods for adequate evaluation (25).

9. For manufactured nanomaterials, specific adaptations of this Test Guideline may be needed but are not described in this Test Guideline.

10. Before using the Test Guideline for testing a mixture to generate data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing the mixture.

11. Mutant cells deficient in thymidine kinase enzyme activity because of a mutation $TK^{+/-}$ to $TK^{-/-}$ are resistant to the cytostatic effects of the pyrimidine analogue trifluorothymidine (TFT). The *TK*

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 and form visible colonies, whereas cells containing the TK enzyme are not.

PRINCIPLE OF THE TEST

12. Cells in suspension are exposed to the test chemical, both with and without an exogenous source of metabolic activation (see paragraph 19), for a suitable period of time (see paragraph 33), and then sub-cultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is determined by relative total growth (RTG—see paragraph 25) for the MLA and by relative survival (RS—see paragraph 26) for TK6. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type (see paragraph 37), to allow near-optimal phenotypic expression of induced mutations. Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

DESCRIPTION OF THE METHOD

Preparations

Cells

13. For MLA: Because the MLA was developed and characterized using the $TK^{+/-}$ -3.7.2C subline of L5178Y cells, this specific subline has to be be used for the MLA. The L5178Y cell line was derived from a methylcholanthrene-induced thymic lymphoma from a DBA-2 mouse (26). Clive and co-workers treated L5178Y cells (designated by Clive as $TK^{+/+}$ -3) with ethylmethane sulfonate and isolated a $TK^{-/-}$ (designated as $TK^{-/-}$ -3.7) clone using bromodeoxyuridine as the selective agent. From the $TK^{-/-}$ clone a spontaneous $TK^{+/-}$ clone (designated as $TK^{+/-}$ -3.7.2.) and a subclone (designated as $TK^{+/-}$-3.7.2C) were isolated and characterized for use in the MLA (27). The karyotype for the cell line has been published (28) (29) (30) (31). The modal chromosome number is 40. There is one metacentric chromosome (t12;13) that should be counted as one chromosome. The mouse *TK* locus is located on the distal end of chromosome 11. The L5178Y $TK^{+/-}$ -3.7.2C cell line has mutations in both p53 alleles and produces mutant-p53 protein (32) (33). The p53 status of the $TK^{+/-}$-3.7.2C cell line is likely responsible for the ability of the test to detect large-scale damage (17).

14. For TK6: The TK6 is a human lymphoblastoid cell line. The parent cell line is an Epstein-Barr virus-transformed cell line, WI-L2, which was originally derived from a 5-year-old male with hereditary spherocytosis. The first isolated clone, HH4, was mutagenized with ICR191 and a *TK* heterozygous cell line, TK6, was generated (34). TK6 cells are nearly diploid and the representative karyotype is 47, XY, 13+, t(14; 20), t(3; 21) (35). The human *TK* locus is located on the long arm of chromosome 17. The TK6 is a p53-competent cell line, because it has a wild-type p53 sequence in both alleles and expresses only wild-type p53 protein (36).

15. For both the MLA and the TK6, when first establishing or replenishing a master stock, it is advisable for the testing laboratory to assure the absence of *Mycoplasma* contamination, karyotype the cells or paint the chromosomes harboring the *TK* locus, and to check population doubling times. The normal cell cycle time for the cells used in the testing laboratory should be established and should be consistent with published cell characteristics (16) (19) (37). This master stock should be stored at -150° C or below and used to prepare all working cell stocks.

16. Either prior to establishing a large number of cryopreserved working stocks or just prior to use in an experiment, the culture may need to be cleansed of pre-existing mutant cells [unless the solvent control mutant frequency (MF) is already within the acceptable range—see Table 2 for the MLA)]. This is accomplished using methotrexate (aminopterin) to select against TK-deficient cells and adding thymidine, hypoxanthine and glycine (L5178Y) or 2'-deoxycytidine (TK6) to the culture to ensure optimal growth of the TK-competent cells (19) (38) (39), and (40) for TK6). General advice on good practice for the maintenance of cell cultures as well as specific advice for L5178Y and TK6 cells can be found in (19) (31) (37) (39) (41). For laboratories requiring master cell stocks to initiate either the MLA or TK6 or to obtain new master cell stocks, a cell repository of well characterized cells is available (37).

Media and culture conditions

17. For both tests, appropriate culture medium and incubation conditions (e.g. culture vessels, humidified atmosphere of 5% CO_2, incubation temperature of 37°C) should be used for maintaining cultures. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. It is particularly important to choose media and culture conditions that ensure optimal growth of cells during the expression period and cloning for both mutant and non-mutant cells. For the MLA and the TK6, it is also important that the culture conditions ensure optimal growth of both the large colony/early appearing and the small colony/late appearing *TK* mutants. More culture details, including the need to properly heat inactivate horse serum if RPMI medium is used during mutant selection can be found in (19) (31) (38) (39) (40) (42).

Preparation of cultures

18. Cells are propagated from stock cultures, seeded in culture medium at a density such that the suspension cultures will continue to grow exponentially through the treatment and expression periods.

Metabolic activation

19. Exogenous metabolizing systems should be used when employing L5178Y and TK6 cells because they have inadequate endogenous metabolic capacity. The most commonly used system that is recommended by default unless otherwise justified, is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 (43) (44) (45) or a combination of phenobarbital and β-naphthoflavone (46) (47) (48) (49) (50) (51). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (52) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (45) (46) (47) (48) (49). The S9 fraction typically is used at concentrations ranging from 1-2% but may be increased to 10% (v/v) in the final test medium. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of test chemicals.

Test chemical preparations

20. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 21). Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels (53) (54) (55). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

Test Conditions

Solvents

21. The solvent should be chosen to optimize the solubility of the test chemical without adversely impacting the conduct of the test, e.g. changing cell growth, affecting the integrity of the test chemical, reacting with culture vessels, impairing the metabolic activation system. It is recommended that, wherever possible, the use of an aqueous solvent (or culture medium) should be considered first. Well established solvents are water or dimethyl sulfoxide. Generally organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If other than well-established solvents are used (e.g. ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemicals, the test system and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to add untreated controls (see Annex 1, Definitions) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Measuring cytotoxicity and choosing treatment concentrations

22. When determining the highest test chemical concentration, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see paragraph 28), precipitation (see paragraph 29) in the culture medium, or marked changes in pH or osmolality (see paragraph 8), should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artifactual positive results and to maintain appropriate culture conditions.

23. Concentration selection is based on cytotoxicity and other considerations (see paragraphs 27-30). While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not required. Even if an initial cytotoxicity evaluation is performed, the measurement of cytotoxicity for each culture is still required in the main experiment. If a range finding experiment is conducted, it should cover a wide range of concentrations and can either be terminated at day 1 after treatment or carried through the 2 day expression and to mutant selection (should it appear that the concentrations used are appropriate).

24. Cytotoxicity should be determined for each individual test culture and control culture: methods for MLA (2) and the TK6 (15) are defined by internationally agreed practice.

25. For both the agar and microwell versions of the MLA: Cytotoxicity should be evaluated using relative total growth (RTG) which was originally defined by Clive and Spector in 1975 (2). This measure includes the relative suspension growth (RSG: test culture vs. solvent control) during the cell treatment, the expression time and the relative cloning efficiency (RCE: test culture vs. solvent control) at the time that mutants are selected (2). It should be noted that the RSG includes any cell loss occurring in the test culture during treatment (See Annex 2 for formulae).

26. For TK6: Cytotoxicity should be evaluated using relative survival (RS) i.e. cloning efficiency of cells plated immediately after treatment, adjusted for any cell loss during treatment, based on cell count as compared to the negative control (assigned a survival of 100%) (See Annex 2 for the formula).

27. At least four test concentrations (not including the solvent and positive controls) that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc) should be evaluated. While the use of duplicate cultures is advisable, either replicate or single treated cultures may be used at each concentration tested. The results obtained for replicate cultures at a given concentration should be reported separately but

can be pooled for the data analysis (55). For test chemicals demonstrating little or no cytotoxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. Where cytotoxicity occurs, concentrations should be selected to cover the cytotoxicity range from that producing cytotoxicity as described in paragraph 28 and including concentrations at which there is moderate and little or no cytotoxicity. Many test chemicals exhibit steep concentration response curves and in order to cover the whole range of cytotoxicity or to study the concentration response in detail, it may be necessary to use more closely spaced concentrations and more than four concentrations, in particular in situations where a repeat experiment is required (see paragraph 70). The use of more than 4 concentrations may be particularly important when using single cultures.

28. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RTG for the MLA, and between 20 and 10% RS for the TK6 (paragraph 67).

29. For poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artifactual effects may result from the precipitate. Because the MLA and TK6use suspension cultures, particular care should be taken to assure that the precipitate does not interfere with the conduct of the test. The determination of solubility in the culture medium prior to the experiment may also be useful.

30. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest (57) (58). When the test chemical is not of defined composition e.g. substance of unknown or variable composition, complex reaction products or biological materials [i.e. Chemical Substances of unknown or Variable Composition (UVCBs)], environmental extracts etc., the top concentration, may need to be higher (e.g. 5 mg/mL), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (59).

Controls

31. Concurrent negative controls (see paragraph 21), consisting of the solvent alone in the treatment medium and handled in the same way as the treatment cultures, should be included for every experimental condition.

32. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify mutagens under the conditions of the test protocol used, the effectiveness of the exogenous metabolic activation system (when applicable), and to demonstrate adequate detection of both small/late appearing and large/early appearing *TK* mutants. Examples of positive controls are given in the table 1 below. Alternative positive control substances can be used, if justified. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized for short-term treatments (3-4 hours) done concurrently with and without metabolic activation using the same treatment duration, the use of positive controls may be confined to a mutagen requiring metabolic activation. In this case, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. If used, long term treatment (i.e. 24 hours without S9) should however have its own positive control, as the treatment duration will differ from the test using metabolic activation. Each positive control should be used at one or more concentrations expected to give reproducible and detectable increases over background in order to demonstrate the sensitivity of the test system, and the response should not be compromised by cytotoxicity exceeding the limits specified in this TG (see paragraph 28).

Table 1: Reference substances recommended for assessing laboratory proficiency and for selection of positive controls

Category	Substance	CASRN
1. Mutagens active without metabolic activation		
	Methyl methanesulphonate	66-27-3
	Mitomycin C	50-07-7
	4-Nitroquinoline-N-Oxide	56-57-5
2. Mutagens requiring metabolic activation		
	Benzo(a)pyrene	50-32-8
	Cyclophosphamide	50-18-0
	(monohydrate)	(6055-19-2)
	7,12-Dimethylbenzanthracene	57-97-6
	3-Methylcholanthrene	56-49-5

PROCEDURE

Treatment with test chemical

33. Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system. Exposure should be for a suitable period of time (usually 3 to 4 hours is adequate). It should be noted however that these requirements may differ for human pharmaceuticals (59). For MLA, in cases where the short-term treatment yields negative results, and there is information suggesting the need for longer treatment [e.g. nucleoside analogs, poorly soluble substances, (5) (59)], consideration should be given to conducting the test with longer treatment, i.e. 24 hours without S9.

34. The minimum number of cells used for each test (control and treated) culture at each stage in the test should be based on the spontaneous mutant frequency. A general guide is to treat and passage sufficient cells in each experimental culture so as to maintain at least 10 but ideally 100 spontaneous mutants in all phases of the test (treatment, phenotypic expression and mutant selection) (56).

35. For MLA the recommended acceptable spontaneous mutant frequency is between 35-140 x 10^{-6} (agar version) and 50-170 x 10^{-6} (microwell version) (see Table 2). To have at least 10 and ideally 100 spontaneous mutants surviving treatment for each test culture, it is necessary to treat at least 6 x 10^{6} cells. Treating this number of cells, and maintaining sufficient cells during expression and cloning for mutant selection, provides for a sufficient number of spontaneous mutants (10 or more) during all phases of the experiment, even for the cultures treated at concentrations that result in 90% cytotoxicity (as measured by an RTG of 10%) (19) (38) (39).

36. For the TK6, the spontaneous mutant frequency is generally between 2 and 10 x 10^{-6}. To have at least 10 spontaneous mutants surviving treatment for each culture, it is necessary to treat at least 20 x 10^{6} cells. Treating this number of cells provides for a sufficient number of spontaneous mutants (10 or more) even for the cultures treated at concentrations that cause 90% cytotoxicity during treatment (10% RS). In addition a sufficient number of cells must be cultured during the expression period and plated for mutant selection (60).

Phenotypic expression time and measurement of cytotoxicity and mutant frequency

37. At the end of the treatment period, cells are cultured for a defined time to allow near optimal phenotypic expression of newly induced mutants; specific to each cell line. For the MLA, the phenotypic expression period is 2 days. For the TK6, the phenotypic expression period is 3-4 days. If a 24 hr treatment is used, the expression period begins after the end of treatment.

38. During the phenotypic expression period, cells are enumerated on a daily basis. For the MLA the daily cell counts are used to calculate the daily suspension growth (SG). Following the 2 day expression period, cells are suspended in medium with and without selective agent for the determination of the numbers of mutants (selection plates) and for cloning efficiency (viability plates), respectively. For MLA there are two equally acceptable methods for mutant selection cloning; one using soft agar and the other using liquid medium in 96-well plates (19) (38) (39). Cloning in the TK6 is conducted using liquid media and 96-well plates (16).

39. Triflurothymidine (TFT) is the only recommended selective agent for *TK* mutants (61).

40. For the MLA, agar plates and microwell plates are counted after 10-12 days incubation. For the TK6, colonies in microwell plates are scored after 10-14 days for the early appearing mutants. In order to recover the slow growing (late appearing) TK6 mutants, it is necessary to re-feed the cells with growth medium and TFT after counting the early appearing mutants and then to incubate the plates for an additional 7-10 days (62). See paragraphs 42 & 44 for a discussion concerning the enumeration of the slow and normal growth *TK* mutants.

41. The appropriate calculations for the two tests including the two methods (agar and microwell) for the MLA are in Annex 2. For the agar method of the MLA, colonies are counted and the number of mutant colonies adjusted by the cloning efficiency to calculate a MF. For the microwell version of the MLA and the TK6, cloning efficiency both for the selection and cloning efficiency plates is determined according to the Poisson distribution (63). The MF is calculated from these two cloning efficiencies.

Mutant Colony characterization

42. For the MLA, if the test chemical is positive (see paragraphs 62-63), colony characterization by colony sizing or growth should be performed on at least one of the test cultures (generally the highest acceptable positive concentration) and on the negative and positive controls. If the test chemical is negative (see paragraph 64), mutant colony characterization should be performed on the negative and positive controls. For the microwell method of the MLA, small colony mutants are defined as those covering less than 25% of the well's diameter and large colony mutants as those that cover more than 25% of the well's diameter. For the agar method, an automatic colony counter is used to enumerate the mutant colonies and for colony sizing. Approaches to colony sizing are detailed in the literature (19) (38) (40). Colony characterization on the negative and positive control is needed to demonstrate that the studies are adequately conducted.

43. The test chemical cannot be determined to be negative if the both the large and small colony mutants are not adequately detected in the positive control. Colony characterization can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events (paragraph 4).

44. TK6: Normal growing and slow growing mutants are differentiated by a difference in incubation time (see paragraph 40). For the TK6 generally both the early and late appearing mutants are scored for all of the cultures including the negative and positive controls. Colony characterization of the negative and

positive control is needed to demonstrate that the studies are adequately conducted. The test chemical cannot be determined to be negative if both the early appearing and late appearing mutants are not adequately detected in the positive control. Colony characterization can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events (paragraph 4).

Proficiency of the laboratory

45. In order to demonstrate sufficient experience with the test prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms (at least one active with and one active without metabolic activation selected from the substances listed in Table 1) and various negative controls (including untreated cultures and various solvents/vehicles). These positive and negative control responses should be consistent with the literature. This requirement is not applicable to laboratories that have experience, i.e. that have an historical data base available as defined in paragraphs 47-50. For the MLA the values obtained for both positive and negative controls should be consistent with the IWGT recommendations (see Table 2).

46. A selection of positive control substances (see Table 1) should be investigated with short and long treatments (if using long treatments) in the absence of metabolic activation, and also with short treatment in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic substances, to determine the effectiveness of the metabolic activation system and to demonstrate the appropriateness of the cell growth conditions during treatment, phenotypic expression and mutant selection and of the scoring procedures. A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

Historical control data

47. The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

48. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published negative control data. As more experimental data are added to the control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution (64) (65).

49. The laboratory's historical negative control database should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (65), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (66). Further details and recommendations on how to build and use the historical data can be found in the literature (64).

50. Negative control data should consist of mutant frequencies from single or preferably replicate cultures as described in paragraph 27. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database. Where negative control data fall outside the 95% control limit they may be acceptable for inclusion in the historical control distribution as long as these data are not extreme outliers, there is evidence that the test system is 'under control' (see paragraph 49) and there is evidence of no technical or human failure.

51. Any changes to the experimental protocol should be considered in terms of the consistency of the data with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

DATA AND REPORTING

Presentation of the results

52. The presentation of data for both the MLA and TK6 should include, for both treated and control cultures, data required for the calculation of cytotoxicity (RTG or RS, respectively) and mutant frequencies, as described below.

53. For MLA, individual culture data should be provided for RSG, RTG, the cloning efficiency at the time of mutant selection and the number of mutant colonies (for agar version) or number of empty wells (for microwell version). MF should be expressed as number of mutant cells per million surviving cells. If the response is positive, small and large colony MFs (and/or percentage of the total MF) should be given for at least one concentration of the test chemical (generally the highest positive concentration) and the negative and positive controls. In the case of a negative response, the small and large colony MF should be given for the negative control and the positive control.

54. For TK6, individual culture data should be provided for RS, the cloning efficiency at the time of mutant selection and the number of empty wells for early appearing and late appearing mutants. MF should be expressed as number of mutant cells per number of surviving cells, and should include the total MF as well as the MF (and/or percentage of the total MF) of the early and late appearing mutants.

Acceptability Criteria

55. For both the MLA and the TK6 the following criteria should be met before determining the overall results for a specific test chemical:

- Two experimental conditions (short treatment with and without metabolic activation - see paragraph 33) were conducted unless one resulted in positive results.

- Adequate number of cells and concentrations should be analysable (see paragraphs 27, 34-36).

- The criteria for the selection of top concentration are consistent with those described in paragraphs 28-30.

Acceptability criteria for negative and positive controls

56. The IWGT Expert MLA Workgroup analysis of an extensive amount of MLA data resulted in international consensus for specific acceptability criteria for the MLA (1) (2) (3) (4) (5). Therefore, this Test Guideline provides specific recommendations for determining the acceptability of negative and positive controls and for evaluating individual substance results in the MLA. The TK6 has a much smaller database and has not undergone evaluation by a workgroup.

57. For MLA**,** every experiment should be evaluated as to whether the untreated/solvent control meets the IWGT MLA Workgroup acceptance criteria ((4) and Table 2, below) for the: (1) MF (note that the IWGT acceptable MFs are different for the agar and microwell versions of the MLA), (2) cloning efficiency (CE) at the time of mutant selection and (3) suspension growth (SG) for the solvent control (see Annex 2 for formulae).

Table 2: Acceptability criteria for the MLA

Parameter	Soft Agar Method	Microwell Method
Mutant Frequency	35 – 140 X 10^{-6}	50 – 170 X 10^{-6}
Cloning Efficiency	65 – 120%	65 – 120%
Suspension Growth	8 – 32 fold (3-4 hour treatment) 32 – 180 fold (24 hour treatment, if conducted)	8 – 32 fold (3-4 hour treatment) 32 – 180 fold (24 hour treatment, if conducted)

58. For MLA, every test should also be evaluated as to whether the positive control(s) meets at least one of the following two acceptance criteria developed by the IWGT workgroup:

(1) The positive control should demonstrate an absolute increase in total MF, that is, an increase above the spontaneous background MF [an induced MF (IMF)] of at least 300 X 10^{-6}. At least 40% of the IMF should be reflected in the small colony MF.
(2) The positive control has an increase in the small colony MF of at least 150 X 10^{-6} above that seen in the concurrent untreated/solvent control (a small colony IMF of 150 X 10^{-6}).

59. For the TK6, a test will be acceptable if the concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraphs 48-49. In addition, the concurrent positive controls (see paragraph 32) should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.

60. For both tests, the upper limit of cytotoxicity observed in the positive control culture should be the same as of the experimental cultures. That is, the RTG/RS should not be less than 10%. It is sufficient to use a single concentration (or one of the concentrations of the positive control cultures if more than one concentration is used) to demonstrate that the acceptance criteria for the positive control have been satisfied. Further, the MF of the positive control must be within the acceptable range established for the laboratory.

Evaluation and interpretation of results

61. For the MLA, significant work on biological relevance and criteria for a positive response has been conducted by The Mouse Lymphoma Expert Workgroup of the IWGT (4). Therefore, this test guideline provides specific recommendations for the interpretation of test chemical results from the MLA (see paragraphs 62-64). The TK6 has a much smaller database and has not undergone evaluation by a workgroup. Therefore, the recommendations for the interpretation of data for the TK6 are given in more general terms (see paragraphs 65-66). Additional recommendations apply to both tests (see paragraphs 67-71).

MLA

62. An approach for defining positive and negative responses is recommended to assure that the increased MF is biologically relevant. In place of statistical analysis generally used for other tests, it relies on the use of a predefined induced mutant frequency (i.e. increase in MF above concurrent control), designated the Global Evaluation Factor (GEF), which is based on the analysis of the distribution of the negative control MF data from participating laboratories (4). For the agar version of the MLA the GEF is 90 x 10^{-6} and for the microwell version of the MLA the GEF is 126 x 10^{-6}.

63. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined (see paragraph 33), the increase in MF above the concurrent background exceeds the GEF and the increase is concentration related (e.g. using a trend test). The test chemical is then considered able to induce mutation in this test system.

64. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly negative if, in all experimental conditions examined (see paragraph 33) there is no concentration related response or, if there is an increase in MF, it does not exceed the GEF. The test chemical is then considered unable to induce mutations in this test system.

TK6

65. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined (see paragraph 33):

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control

b) the increase is concentration-related when evaluated with an appropriate trend test (see paragraph 33)

c) any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 48).

When all of these criteria are met, the test chemical is then considered able to induce mutation in this test system. Recommendations for the most appropriate statistical methods can be found in the literature (66) (67).

66. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined (see paragraph 33):

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test

c) all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 48).

The test chemical is then considered unable to induce mutations in this test system.

For both the MLA and TK6:

67. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RTG/RS. The consensus is that care should be taken when interpreting positive results only found between 20 and 10% RTG/RS and a result would not be considered positive if the increase in MF occurred only at or below 10% RTG/RS (if evaluated) (2) (59).

68. There are some circumstances under which additional information may assist in determining that a test chemical is not mutagenic when there is no culture showing an RTG value between 10-20 % RTG/RS. These situations are outlined as follows: (1) There is no evidence of mutagenicity (e.g. no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points within 100% to 20% RTG/RS and there is at least one data point between 20 and 25% RTG/RS. (2) There is no evidence of mutagenicity (e.g. no dose response,

no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points between 100% to 25% RTG/RS and there is also a negative data point slightly below 10% RTG/RS. In both of these situations the test chemical can be concluded to be negative.

69. There is no requirement for verification of a clearly positive or negative response.

70. In cases when the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions [e.g. concentration spacing to increase the probability of attaining data points within the 10-20% RTG/RS range, using other metabolic activation conditions (i.e. S9 concentration or S9 origin) and duration of treatment] could be useful.

71. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

Test Report

72. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.
- Multi-constituent substance, UVBCs and mixtures:
- characterized as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent:

- justification for choice of solvent;
- percentage of solvent in the final culture medium.

Cells:

For Laboratory master cultures:

- type and source of cells, and history in the testing laboratory;
- karyotype features and/or modal number of chromosomes;
- methods for maintenance of cell cultures;
- absence of mycoplasma;
- cell doubling times.

Test conditions:

- rationale for selection of concentrations and number of cell cultures; including e.g. cytotoxicity data and solubility limitations;

- composition of media, CO_2 concentration, humidity level;
- concentration of test chemical expressed as final concentration in the culture medium (e.g. µg or mg/mL or mM of culture medium);
- concentration (and/or volume) of solvent and test chemical added in the culture medium;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density during treatment;
- type and composition of metabolic activation system (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix and S9 in the final culture medium, quality controls of S9);
- positive and negative control substances, final concentrations for each conditions of treatment;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- identity of the selective agent and its concentration;
- for the MLA, the version used (agar or microwell) should be indicated
- criteria for acceptability of the tests;
- methods used to enumerate numbers of viable and mutant cells;
- methods used for the measurements of cytotoxicity;
- any supplementary information relevant to cytotoxicity and method used;
- duration of incubation times after plating;
- definition of colonies of which size and type are considered (including criteria for "small' and "large" colonies, as appropriate);
- criteria for considering studies as positive, negative or equivocal;
- methods used to determine pH, osmolality, if performed and precipitation if relevant.

Results:

- number of cells treated and number of cells sub-cultured for each culture;
- toxicity parameters (RTG for MLA and RS for TK6);
- signs of precipitation and time of the determination;
- number of cells plated in selective and non-selective medium;
- number of colonies in non-selective medium and number of resistant colonies in selective medium and related mutant frequencies;
- colony sizing for the negative and positive controls and if the test chemical is positive, at least one concentration, and related mutant frequencies;
- concentration-response relationship, where possible;
- concurrent negative (solvent) and positive control data (concentrations and solvents);
- historical negative (solvent) and positive control data (concentrations and solvents) with ranges, means and standard deviations; number of tests upon which the historical controls are based;
- statistical analyses (for individual cultures and pooled replicates if appropriate), and p-values if any; and for the MLA, the GEF evaluation.

Discussion of the results.

Conclusion..

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ANNEX 1:

DEFINITIONS

Aneugen: Any substance or process that, by interacting with the components of the mitotic and meiotic cell division cycle, leads to aneuploidy in cells or organisms.

Aneuploidy: Any deviation from the normal diploid (or haploid) number of chromosomes by a single chromosome or more than one, but not by entire set(s) of chromosomes (polyploidy).

Base-pair-substitution mutagens: Substances that cause substitution of base pairs in the DNA.

Cloning efficiency: The percentage of cells plated at a low density that are able to grow into a colony that can be counted.

Clastogen**:** Any substance or process which causes structural chromosomal aberrations in populations of cells or organisms.

Cytotoxicity: For the assays covered in this guideline, cytotoxicity is identified as a reduction in relative total growth (RTG) or relative survival (RS) for the MLA and TK6, respectively.

Forward mutation: A gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens: Substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Genotoxic: A general term encompassing all types of DNA or chromosomal damage, including DNA breakage, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosomal damage.

Mitotic recombination: During mitosis, recombination between homologous chromatids possibly resulting in the induction of DNA double strand breaks or in a loss of heterozygosity.

Mutagenic: Produces a heritable change of DNA base-pair sequences(s) in genes or of the structure of chromosomes (chromosome aberrations).

Mutant frequency (MF): The number of mutant cells observed divided by the number of viable cells.

Phenotypic expression time: The time after treatment during which the genetic alteration is fixed within the genome and any preexisting gene products are depleted to the point that the phenotypic trait is altered.

Relative survival (RS): RS is used as the measure of treatment-related cytotoxicity in the TK6. It is the relative cloning efficiency (CE) of cells plated immediately after the cell treatment adjusted by any loss of cells during treatment as compared with the cloning efficiency of the negative control.

Relative suspension growth (RSG): For the MLA, the relative total two day suspension growth of the test culture compared to the total two-day suspension growth of the negative/solvent control (Clive and

Spector, 1975). The RSG should include the relative growth of the test culture compared to the negative/solvent control during the treatment period.

Relative total growth (RTG): RTG is used as the measure of treatment-related cytotoxicity in the MLA. It is a measure of relative (to the vehicle control) growth of test cultures during the, treatment, two-day expression and mutant selection cloning phases of the test. The RSG of each test culture is multiplied by the relative cloning efficiency of the test culture at the time of mutant selection and expressed relative to the cloning efficiency of the negative/solvent control (Clive and Spector, 1975).

S9 liver fractions: Supernatant of liver homogenate after 9000g centrifugation, i.e. raw liver extract

S9 mix: Mix of the liver S9 fraction and cofactors necessary for metabolic enzyme activity.

Suspension growth (SG): The fold-increase in the number of cells over the course of the treatment and expression phases of the MLA. The SG is calculated by multiplying the fold-increase on day 1 by the fold-increase on day 2 for the short (3 or 4 hr) treatment. If a 24 hr treatment is used the SG is the fold-increase during the 24 hr treatment multiplied by the fold increases on expression days 1 and 2.

Solvent control**:** General term to define the control cultures receiving the solvent alone used to dissolve the test chemical.

Untreated controls: Untreated controls are cultures that receive no treatment (i.e. no test chemical nor solvent) but are processed the same way as the cultures receiving the test chemical.

Annex 2:

FORMULAS

Cytotoxicity

For both versions (agar and microwell) of the MLA
Cytotoxicity is defined as the Relative Total Growth (RTG) which includes the Relative Suspension Growth (RSG) during the 2 day expression period and the Relative Cloning Efficiency (RCE) obtained at the time of mutant selection. RTG, RSG and RCE are all expressed as a percentage.

Calculation of RSG: Suspension Growth one (SG_1) is the growth rate between day 0 and day 1 (cell concentration at day 1 / cell concentration at day 0) and Suspension Growth two (SG_2) is the growth rate between day 1 and day 2 (cell concentration at day 2 / cell concentration at day 1). The RSG is the total SG (SG_1 X SG_2) for the treated culture compared to the untreated/solvent control. That is: RSG = $[SG_{1(test)} \times SG_{2(test)}] / [SG_{1(control)} \times SG_{2(control)}]$ The SG_1 should be calculated from the initial cell concentration used at the beginning of cell treatment. The accounts for any differential cytotoxicity that occurs in the test culture(s) during the cell treatment.

RCE is the relative cloning efficiency of the test culture compared to the relative cloning efficiency of the untreated/solvent control obtained at the time of mutant selection.

Relative Total Growth (RTG): RTG=RSG x RCE

TK6

Relative Survival (RS):
Cytotoxicity is evaluated by relative survival, i.e. cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with cloning efficiency in the negative controls (assigned a survival of 100%). The adjustment for cell loss during treatment can be calculated as:

$$\text{Adjusted CE} = \text{CE} \times \frac{\text{Number of cells at the end of treatment}}{\text{Number of cells at the beginning of treatment}}$$

The RS for a culture treated by a test chemical is calculated as:

$$\text{RS} = \frac{\text{Adjusted CE in the treated culture}}{\text{Adjusted CE in the solvent control}} \times 100$$

Mutant frequency for both the MLA and TK6

Mutant frequency (MF) is the cloning efficiency of mutant colonies in selective medium (CE_M) adjusted by the cloning efficiency in non-selective medium at the time of mutant selection (CE_V). That is, $MF=CE_M/CE_V$. The calculation of these two cloning efficiencies is described below for the agar and microwell cloning methods.

MLA Agar Version*:* In the soft agar version of the MLA, the number of colonies on the mutant selection plate (C_M) and number of colonies on the unselected or cloning efficiency (viable count) plate (C_V) are obtained by directly counting the clones. When 600 cells are plated for cloning efficiency (CE) for the

mutant selection (CE_M) plates and the unselected or cloning efficiency (viable count) plates (CE_V) and 3 x 10^6 cells are used for mutant selection,

$$CE_M = C_M / (3 \times 10^6) = (C_M / 3) \times 10^{-6}$$
$$CE_V = C_V / 600$$

MLA and TK6 Microwell Version: In the microwell version of the MLA, C_M and C_V are determined as the product of the total number of microwells (TW) and the probable number of colonies per well (P) on microwell plates.

$$C_M = P_M \times TW_M$$
$$CV = P_V \times TW_V$$

From the zero term of the Poisson distribution (Furth et al., 1981), the P is given by

$$P = - \ln (EW / TW)$$

Where, EW is empty wells and TW is total wells. Therefore,

$$CE_M = C_M / T_M = (P_M \times TW_M) / T_M$$
$$CE_V = C_V / T_V = (P_V \times TW_V) / T_V$$

For the microwell version of the MLA, small and large colony mutant frequencies will be calculated in an identical manner, using the relevant number of empty wells for small and large colonies.

For TK6, small and large colony mutant frequencies are based on the early appearing and late appearing mutants.