INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs and animal welfare considerations and revised as appropriate. This test guideline is part of a series of test Guidelines on genetic toxicology. A document presented as an introduction to the Test Guidelines on genetic toxicology (OECD 201x) can also be referred to and provides succinct and useful guidance to supplement these test guidelines. The mouse lymphoma (MLA) and TK6 assays 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 parameter acceptance criteria and data interpretation for the MLA (Moore et al. 2000, 2002, 2003, 2006, 2007), and these recommendations are being incorporated into this new test guideline (TGxxx). TGxxx is written for the MLA and, because it also utilizes the TK locus, the TK6 assay. The MLA has been widely used in a large number of laboratories, for regulatory purposes and has an extensive literature (see Literature List). The TK6 assay has been used much less frequently and has a much smaller literature (see Literature List). It should be noted that in spite of the similarity of the assay 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. Because the MLA and TK6 are now covered by this new TG, as a part of the revision process, TG476 (approved 2015) now includes only the assays using the endogeneous hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene and the much less frequently used xanthine-guanine phosphoribosyl transferase (XPRT) transgenic AS52 line of CHO cells.

2. Although the proper nomenclature is TK for humans and Tk for rodents, TK is used in this guideline for convenience. TK assays can be used to detect mutations induced by chemical substances and physical agents. 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 assays can be conducted in a similar way in both cell types as described in this guideline.

3. The Test Guideline is organized so as to provide general information that applies to both MLA and TK6 and specialized guidance for the individual assays.

4. 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 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 assays cannot reliably detect aneugens when applying standard cytotoxicity criteria (as described in this guideline) and therefore, it is not appropriate to use these assays to detect aneugens (Fellows et al, 2012, Honma et al, 2001 and Wang et al, 2009).

5. In the TK gene mutation assays, 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 assay, respectively. The molecular and cytogenetic nature of both large and small colony MLA mutants has been explored in detail. (Applegate, et al, 1990, Honma, et. al., 2001, Hozier et al., 1981, 1985, Moore et al., 1985; and Wang et al, 2009). The molecular and cytogenetic nature of the early appearing and late appearing TK6 mutants has also been extensively investigated (Liber et.al., 1987, Li et.al., 1992, Honma et al., 1997, 2000). 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 (Amundson and Liber, 1992). 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 substance (Applegate, et al., 1990, Amundson and Liber, 1992, Moore et al., 1985, Schisler et al., 2013).

6. Definitions used are provided in the Annex.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. Assays conducted using L5178Y TK⁺⁻⁻⁻⁻⁻ or TK6 cells 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 substance and the genetic material of the cell; such conditions include changes in pH or osmolality, interaction with the medium components (Long, et al, 2007, Nesslany et al., 2008), or excessive levels of cytotoxicity (Brusick 1986, Morita et al., 1992 and Scott, et al., 1991). Excessive cytotoxicity for the MLA and TK6 assays would be that exceeding the recommended top cytotoxicity levels as defined in paragraph 31. 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 (Wang et al., 2007).

9. For MLA there are two equally acceptable methods for mutant selection cloning; one using soft agar and the other using liquid medium in 96-microwell plates (Lloyd and Kidd., 2012, Mei et al., 2014 and Schisler et al., 2013). The TK6 assay is conducted using liquid media cloning in 96-microwell plates (Honma et al., 1997).

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

11. 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.

PRINCIPLE OF THE TEST

12. Cells in suspension are exposed to the test substance, both with and without an exogenous source of metabolic activation (see paragraph 22), for a suitable period of time (see paragraph 36). The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type, to allow optimal phenotypic expression prior to mutant selection (see paragraph 40).
13. Cells deficient in thymidine kinase enzyme activity due to the 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.

**DESCRIPTION OF THE METHOD**

**Preparations**

**Cells**

14. For MLA: Because the assay was developed and characterized using the $TK^{+/-}$-3.7.2C subline of L5178Y cells, this specific subline must be used for the MLA. The L5178Y cell line was derived from a methylcholanthrene-induced thymic lymphoma from a DBA-2 mouse (Fischer, 1958). 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.) was isolated. Subsequently, a subclone designated as $TK^{+/-}$-3.7.2C was isolated and characterized for use in a mutation assay (Clive et al., 1979). The karyotype for the cell line has been published (Sawyer, et al., 1985, 1989, 2006; Fellows et al., 2013). 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 (Storer et al., 1997, Clark et al. 2004). The p53 status of the MLA is likely responsible for the ability of the assay to detect large-scale damage which may be incompatible with the survival of Trp53-sufficient cells which will undergo apoptosis under the surveillance of Trp53 (Honma et al., 2000).

15. For TK6 assay: 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 (Skopek et al., 1978). TK6 cells are nearly diploid and the representative karyotype is 47, XY, 13+, t(14; 20), t(3; 21) (Honma, 2005). 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 (Xia et al., 1995).

16. For both assays, when first establishing or replenishing a master stock, it is advisable for all laboratories to assure the absence of Mycoplasma contamination, karyotype the cells or paint the chromosomes harboring the $TK$ locus, and to check population doubling times. This master stock should be stored at -150°C or below and used to prepare all working cell stocks. 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 (amifopterin) to select against TK-deficient cells and thymidine, hypoxanthine and glycine (L5178Y) or 2’-deoxycytidine (TK6) are added to the culture to ensure optimal growth of the TK-competent cells. Details for the cell cleansing procedure for the MLA can be found in Lloyd and Kidd, 2012, Mei et al., 2014, Schisler et al., 2013, and for TK6 in Liber and Thilly, 1982. The cleansed cells can be cryopreserved in a large number of individual vials and then thawed to use as working stocks for the TK mutation assay after normal doubling times are attained.

17. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. The normal cell cycle time for the cells used in the testing laboratory should be established and should be consistent with published cell characteristics. (Honma et al., 1997, Lorge et al., in prep., Schisler et al., 2013). General advice on good practice for the maintenance of cell cultures as well as specific advice for L5178Y and TK6 cells is provided in (Coecke et al., Lorge et al., in prep., Fellows, 2013, Schisler et al., 2013; Mei et al., 2014). For laboratories requiring master cell stocks to initiate either the MLA or TK6 assays or to obtain new master cell stocks, a cell repository of well characterized cells is available (Lorge et al., in prep.).

18. Working stocks should be checked with each sub-culturing for population doubling times, and cells should not be used if the population doubling time is unacceptable. In addition, for both assays, the specific cells used in an experiment should demonstrate an acceptable cloning efficiency and an acceptable solvent control MF (see paragraphs 58, 59, 61 and Table 2).

**Media and culture conditions**

19. For both assays, appropriate culture medium and incubation conditions (e.g. culture vessels, humidified atmosphere of 5% CO₂, incubation temperature of 37°C) should be used for maintaining cultures. 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 assay, 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.

20. For the MLA, both Fischer's medium for leukemic cells of mice and RPMI 1640 media have been successfully used along with horse serum (Lloyd and Kidd, 2012, Mei et al., 2014, Schisler, et al., 2013). For the TK6 assay, generally RPMI medium and horse serum are used. (Liber and Thilly, 1982). If RPMI medium is used during the mutant selection it is critical that the horse serum be properly heat inactivated. Inadequate heat inactivation of serum may result in the recovery of clones that are not actually TK mutants (Moore and Howard, 1982, and Fellows et al., 2013)

**Preparation of cultures**
21. Cells are propagated from stock cultures, seeded in culture medium at a density such that suspension cultures will continue to grow exponentially.

*Metabolic activation*

22. 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 (Ames et al., 1975, Maron and Ames, 1983, Natarajan et al., 1976) or a combination of phenobarbitone and β-naphthoflavone (Matsuoka et al., 1979, Ong et al., 1980, Elliott et al., 1992, Matsushima, et al., 1976, Galloway et al., 1994, Johnson et al., 1996). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (UNEP, 2001) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (Matsuoka et al., 1979, Ong et al., 1980, Elliott et al., 1992, Galloway et al., 1994). 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 substances being tested. For a more detailed discussion on the use of metabolizing systems see the Introduction document (OECD, xxx).

*Test chemical preparations*

23. Solid test substances should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 24). Liquid test substances 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 (Krahn et al., 1982; Zamora et al. 1983; Asakura, 2008). Preparations of the test substance should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

*Test Conditions*

*Solvents*

24. The solvent should be chosen to optimize the solubility of the test substance without adversely impacting the conduct of the assay, e.g., changing cell growth, affecting the integrity of the test substance, 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 substances, the test system and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to include untreated controls (see Annex 1, Definitions), as well as solvent controls to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Measuring cytotoxicity and choosing treatment concentrations

25. When determining the highest test substance concentration, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see paragraph 31), precipitation (see paragraph 32) in the culture medium, or marked changes in pH or osmolality (see paragraph 8), should be avoided. If the test substance 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.

26. Concentration selection is based on cytotoxicity and other considerations (see paragraphs 30-33). While the evaluation of cytotoxicity in an initial assay may be useful to better define the concentrations to be used in the main experiment, an initial assay 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).

27. Cytotoxicity should be determined for each individual test culture and control culture. The methods for MLA (Moore et al., 2002) and the TK6 assay (Honma et al., 1997) are defined by internationally agreed practice.

28. For both the agar and microwells versions of the MLA: Cytotoxicity should be evaluated using relative total growth (RTG) which was originally defined by Clive and Spector in 1975 (Moore et al., 2002). 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 (Moore et al. 2002). It should be noted that the RSG includes any cell loss occurring in the test culture during treatment. (See Annex 2 for formulae).

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

30. 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 (Arlett et al., 1989). For test substances 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 31 and including concentrations at which there is moderate and little or no cytotoxicity. Many test substances 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 71). The use of more than 4 concentrations may be particularly important when using single cultures.

31. 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 assay.

32. For poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analyzed 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 TK assays use suspension cultures, particular care should be taken to assure that the precipitate does not interfere with the conduct of the assay. The determination of solubility in the culture medium prior to the experiment may also be useful.

33. 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 (OECD, 2013, Morita et al., 2012, Brookmire et al., 2013). When the test substance 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) (EPA, 2011)], 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 (ICH).

Controls

34. Concurrent negative controls (see paragraph 24), 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.
35. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify mutagens under the conditions of the assay protocol used, the effectiveness of the exogenous metabolic activation system (when applicable), and to demonstrate adequate detection of both small/slow and large/normally growing 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 assays 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 assay 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 assay 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 assay system, and the response should not be compromised by cytotoxicity exceeding the limits specified in this TG (see paragraph 31).

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

<table>
<thead>
<tr>
<th>Metabolic Activation Condition</th>
<th>Substance</th>
<th>CASRN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of exogenous metabolic activation</td>
<td>Mitomycin C</td>
<td>50-07-7</td>
</tr>
<tr>
<td></td>
<td>Methyl methanesulfonate</td>
<td>66-37-3</td>
</tr>
<tr>
<td></td>
<td>4-Nitroquinoline-N-Oxide</td>
<td>56-57-5</td>
</tr>
<tr>
<td>Presence of exogenous metabolic activation</td>
<td>3-Methylcholanthrene</td>
<td>56-49-5</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>50-32-8</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide (monohydrate)</td>
<td>50-18-0 (6055-19-2)</td>
</tr>
<tr>
<td></td>
<td>7,12-Dimethylbenzanthracene</td>
<td>57-97-6</td>
</tr>
</tbody>
</table>
PROCEDURE

Treatment with test chemical

36. Proliferating cells are treated with the test substance 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 (ICH). 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, (ICH, Moore et al., 2007)], consideration should be given to conducting the assay with longer treatment, i.e., 24 hours without S9.

37. The number of cells used for each test (control and treated) culture should be based on the normal range of 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 assay (treatment, phenotypic expression and mutant selection) (Arlett et al., 1989).

38. 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%). (Lloyd and Kidd, 2012, Mei et al. 2014, Schisler et al. 2013).

39. For the TK6 assay, 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 (Honma and Hayashi 2011).

Phenotypic expression time and measurement of cytotoxicity and mutant frequency

40. 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 assay, the phenotypic expression period is 3-4 days. If a 24 hr treatment is used, the expression period begins after the end of treatment.
41. 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.

42. Trifluothymidine (TFT) is the only recommended selective agent for TK mutants (Moore-Brown et al. 1981).

43. For the MLA, agar plates and microwell plates are counted after 10-12 days incubation. For the TK6 assay, 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. See paragraphs 45-47 for a discussion concerning the enumeration of the slow and normal growth TK mutants.

44. The appropriate calculations for the two assays including the two methods (agar and microwell) for the MLA are in the 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 assay, cloning efficiency both for the selection and cloning efficiency plates is determined according to the Poisson distribution (Furth et al., 1981). The MF is calculated from these two cloning efficiencies.

Mutant Colony characterization

45. For the MLA, if the test substance is positive (see paragraphs 64-65), 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 substance is negative (see paragraph 66), 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 (Lloyd et al., 2012, Mei et al., 2014, Schisler et al., 2013). Colony characterization on the negative and positive control is needed to demonstrate that the studies are adequately conducted.

46. The test substance 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 substance to cause point mutations and/or chromosomal events (paragraph 5).
47.  TK6 assay: Normal growing and slow growing mutants are differentiated by a difference in incubation time (see paragraph 43). For the TK6 assay 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 substance 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 substance to cause point mutations and/or chromosomal events (paragraph 5).

**Proficiency of the laboratory**

48.  In order to demonstrate sufficient experience with the assay 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 50-52. For the MLA the values obtained for both positive and negative controls should be consistent with the IWGT recommendations (see Table 2).

49.  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, determine the effectiveness of the metabolic activation system and demonstrate the appropriateness of the treatment, expression and mutant selection growth conditions and 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 assay system.

**Historical control data**

50.  The laboratory should establish:

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

51.  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 (Hayashi et al., 2011 and Ryan, 2000). 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 assay system is ‘under control’ (see paragraph 52) and there is evidence of absence of technical or human failure.

52. 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 (Ryan 2000), to identify how variable their positive and negative control data are, and to show that the methodology is ‘under control’ in their laboratory (OECD xx). Further recommendations on how to build and use the historical data (i.e. criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (Hayashi et al., 2011).

53. 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

54. The presentation of data for both the MLA and TK6 assay 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.

55. 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 number of 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 substance (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.

56. For TK6 assay, 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

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

- Two experimental conditions (short treatment with and without metabolic activation) were conducted unless one resulted in positive results (see paragraph 36)

- Adequate number of cells and concentrations should be analysable (see paragraphs 30, 38 and 39).

- The criteria for the selection of top concentration are consistent with those described in paragraphs 31-33.

Acceptability criteria for negative and positive controls

58. The IWGT Expert MLA Workgroup analysis of an extensive amount of MLA data resulted in international consensus for specific assay acceptability criteria for the MLA (Moore et al., 2000, 2002, 2003, 2006, 2007). 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 assay has a much smaller database and has not undergone evaluation by a workgroup.

59. For MLA, every experiment should be evaluated as to whether the untreated/solvent control meets the IWGT MLA Workgroup acceptance criteria (Moore et al., 2006 and Table 2, below) for the: (1) MF (note that the IWGT acceptable MFs are different for the agar and microwell versions of the assay), (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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Soft Agar Method</th>
<th>Microwell Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Frequency</td>
<td>35 – 140 X 10^6</td>
<td>50 – 170 X 10^6</td>
</tr>
<tr>
<td>Cloning Efficiency</td>
<td>65 – 120%</td>
<td>65 – 120%</td>
</tr>
<tr>
<td>Suspension Growth</td>
<td>8 – 32 fold (3-4 hour treatment)</td>
<td>32 – 180 fold (24 hour treatment, if conducted)</td>
</tr>
</tbody>
</table>
For MLA, every assay should also be evaluated as to whether the positive control(s) meets either 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 $300 \times 10^{-6}$. 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 $150 \times 10^{-6}$ above that seen in the concurrent untreated/solvent control (a small colony IMF of $150 \times 10^{-6}$).

For the TK6 assay, an assay will be acceptable if the concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraph 51. The concurrent positive controls (see paragraph 35) 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.

For both assays, 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

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 (Moore et al., 2006). Therefore, this test guideline provides specific recommendations for the interpretation of test substance results from the MLA (see paragraphs 64-66). The TK6 assay has a much smaller database and has not undergone evaluation by a workgroup. Therefore, the recommendations for the interpretation of data for the TK6 assay are given in more general terms (see paragraphs 67-68). Additional recommendations apply to both assays (see paragraphs 69-73).

MLA

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 assays, 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 (Moore et al., 2006). For the agar version of the assay the GEF is $90 \times 10^{-6}$ and for the microwell version of the assay the GEF is $126 \times 10^{-6}$.

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 36), the increase in MF above the concurrent background exceeds the GEF and the increase is concentration related. The test chemical is then considered able to induce mutation in this assay system.

66. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly negative if, in all experimental conditions examined (see paragraph 36) the increase in MF does not exceed the GEF and there is not a concentration related response. The test chemical is then considered unable to induce mutations in this assay system.

**TK6 assay**

67. 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 36):

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 in at least one experimental condition when evaluated with an appropriate trend test (see paragraph 36)

c) any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 51).

When all of these criteria are met, the test chemical is then considered able to induce mutation in this assay system. Recommendations for the most appropriate statistical methods can also be found in the literature (OECD, 2014, Fleiss et al., 2003).

68. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined (see paragraph 36):

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 51).

The test chemical is then considered unable to induce mutations in this assay system. Recommendations for the most appropriate statistical methods can also be found in the literature (OECD, 2014, Fleiss et al., 2003).
For both the MLA and TK6 assays:

69. 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) (ICH, Moore et al., 2002). There are some circumstances under which additional information may assist in determining that a 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% and there is also a negative data point slightly below 10% RTG/RS. In both of these situations the chemical can be concluded to be negative.

70. Verification of a clear positive or negative response is usually not required providing all assay acceptance criteria are fulfilled.

71. 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.

72. There are cases, even after further investigations where 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).

73. An increase in mutant frequency in TK gene mutation assays indicates that the test substance induces viable genetic events affecting the TK gene in the cultured mammalian cells used. Negative results indicate that, under the test conditions, the test substance does not induce such events in the cultured mammalian cells used.

Test Report

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;
- reactivity of the test substances with the solvent/vehicle or cell culture media;
- 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;
- solubility and stability of the test substance in solvent, if known.
- percentage of solvent in the final culture medium should be indicated.

**Cells:**

For Laboratory master cultures:

- 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, CO₂ concentration, humidity level;
- concentration of test substance 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 substance 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);
- selective agent and concentration;
- for the MLA the version used (agar or microwell) should be indicated
- criteria for considering assays as positive, negative or equivocal;
- methods used to enumerate numbers of viable and mutant cells.
- definition of colonies of which size and type are considered (including criteria for "small" and "large" colonies, as appropriate);
- methods used to determine pH, osmolality, if performed and precipitation if relevant

Results:

- the number of cells treated and the number of cells cloned for each culture;
- toxicity parameters (RTG for MLA and RS for TK6);
- signs of precipitation and time of the determination;
- data on pH and osmolality under the conditions of treatment with the test chemical, if determined;
- number of colonies in non-selective medium and number of resistant colonies in selective medium
- colony sizing for the negative and positive controls and if the test chemical is positive, at least one concentration
• concentration-response relationship, where possible;
• statistical analyses (for individual cultures and pooled replicates if appropriate) p-values if any;
• concurrent negative/solvent and positive control data;
• historical negative (negative/solvent) and positive control data with ranges, means and standard deviations; number of assays upon which the historical controls are based;
• spontaneous MF and induced MF;
• raw data, including cell culture counts and colony counts

Discussion of the results.

Conclusion.
LITERATURE  (Note: needs to be formatted)


Sandra Coecke, Michael Balls, Gerard Bowe, John Davis, Gerhard Gstraunthaler, Thomas Hartung, Robert Hay, Otto-Wilhelm Merten, Anna Price, Leonard Schechtman, Glyn Stacey and William


Fellows, M.D., A. McDermott, K.R. Clare, A. Doherty and M.J. Aardema (2013), The spectral karyotype of L5178Y TK+- mouse lymphoma cells clone 3.7.2C and factors affecting mutant frequency at the thymidine kinase (tk) locus in the microtitre mouse lymphoma assay,


International Conference on Harmonisation (ICH) Guidance S2 (R1) on Genotoxicity Testing and Data Interpretation For Pharmaceuticals Intended For Human Use.


OECD (201x), Draft Introduction to the OECD guidelines on genetic toxicology testing and guidance on the selection and application of assays

OECD (2013) Document supporting the WNT decision to implement revised criteria for the selection of the top concentration in the in vitro mammalian cell assays on genotoxicity (Test Guidelines 473, 476 and 487) Olis cote:to be added


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 one or a small number 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 chromosome damage, including DNA breakage, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosome 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 assay. 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 assay. 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 24hr 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 substance.

**Untreated controls:** Untreated controls are cultures that receive no treatment (i.e. no test substance nor solvent) but are processed the same way as the cultures receiving the test substance.
Annex 2:

FORMULAE

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 (SG₁) is the growth rate between day 0 and day 1 (cell concentration at day 1 / cell concentration at day 0) and Suspension Growth two (SG₂) 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 (SG₁ X SG₂) for the treated culture compared to the untreated/solvent control. That is: \( \text{RSG} = \frac{\text{SG}_1(\text{test}) \times \text{SG}_2(\text{test})}{\text{SG}_1(\text{control}) \times \text{SG}_2(\text{control})} \) The SG₁ 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.

\[ \text{Relative Total Growth (RTG)}: \quad \text{RTG} = \text{RSG} \times \text{RCE} \]

TK6 Assay

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:

\[ \text{Adjusted} = \frac{\text{CE} \times \text{No. cells at the end of treatment}}{\text{No. cells at the beginning of treatment}} \]

The RS for a culture treated by a test substance 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 is the cloning efficiency of mutant colonies in selective medium adjusted by the cloning efficiency in non-selective medium at the time of mutant selection.

**MLA Agar Version:** In the soft agar version of the assay, the number of colonies on the mutant selection plate (CM) and number of colonies on the unselected or cloning efficiency (viable count) plate (CV) are obtained by directly counting the clones. When 600 cells are plated for cloning efficiency (CE) for the mutant selection (CM) plates and the unselected or cloning efficiency (viable count) plates (CV) and 3 x 10^6 cells are used for mutant selection,

\[
CE_M = \frac{C_M}{3 \times 10^6} = \left(\frac{C_M}{3}\right) \times 10^{-6}
\]

\[
CE_V = \frac{C_V}{600}
\]

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

\[
CM = P_M \times TW_M
\]

\[
CV = P_V \times TW_V
\]

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

\[
P = -\ln \left(\frac{EW}{TW}\right)
\]

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

\[
CE_M = \frac{C_M}{T_M} = \left(\frac{P_M \times TW_M}{T_M}\right)
\]

\[
CE_V = \frac{C_V}{T_V} = \left(\frac{P_V \times TW_V}{T_V}\right)
\]

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.