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Toxicological Principles for the Safety Assessment of Food Ingredients

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IV.C.1.c. Mouse Lymphoma Thymidine Kinase Gene Mutation Assay

I. Introduction

An *in vitro* mammalian cell gene mutation test can be used to detect gene alterations induced by chemical substances. While there are a number of cell lines that can be used, the L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma cell line using the thymidine kinase (TK) gene is the cell line and assay of choice. The mouse lymphoma assay (MLA) was chosen because of a body of research indicating that many types of genetic alterations are detected. The assay detects mutations known to be important in the etiology of cancer and other human genetically mediated illnesses. There is evidence that the assay detects gene mutations (point mutations) and chromosomal events (deletions, translocations, mitotic recombination/gene conversion and aneuploidy) (Applegate et. al., 1990; Hozier, et al., 1981; Moore, et al., 1985; Sawyer, et al., 1989; Sawyer, et al., 1985). The efficiency of detection of all of these mutational events is still under investigation.

Other *in vitro* mammalian gene mutation assays exist including those that use either Chinese hamster cell lines (CHO, AS52, and V79) or human lymphoblastoid cells (TK6). In these cell lines the most commonly used genetic endpoints measure mutation at either the hypoxanthine-guanine phosphoribosyl transferase (HPRT), a transgene of xanthine-guanine phosphoribosyl transferase (XPRT), or TK. The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK allows for the detection of genetic events that are not detected at the HPRT locus on the X-chromosome (Moore et al., 1989).

The various mutation assays are capable of detecting different spectra of genetic damage. Thus, it is not expected that a chemical will give uniformly positive or negative results in the various assays. In particular, the bacterial Salmonella assay detects only point and other very small-scale gene mutations. Furthermore, the *in vitro* mammalian assays using the HPRT locus are unable to detect chemicals that do not induce point mutations yet are clastogenic (Moore et. al, 1989). These chemicals are likely operating by mechanisms that cause chromosomal mutations

(deletions, translocations, gene conversion/mitotic recombination and/or aneuploidy).

Additional information is provided in Chapter IV. C. 1.

II. Definitions

Forward mutation: A mutation that converts a wild-type allele to a mutant allele.

Base-pair-substitution mutagens: Substances that cause substitution of one or a small number of base pairs in the DNA.

Frameshift mutagens: Substances that cause insertion or deletion of a nucleotide pair or pairs, causing a disruption of the triplet translational reading frame.

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.

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

Relative survival (RS): The relative cloning efficiency of the test culture plated immediately after the cell treatment and compared to the cloning efficiency of the negative control (Cole, et al., 1986)

Relative suspension growth (RSG): The relative total two day suspension growth of the test culture compared to the total two-day suspension growth of the vehicle control. (Clive and Spector, 1975).

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 both during the 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 vehicle control.

III. Initial Considerations

The established cell line L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma is used for the assay. The assay requires the use of an exogenous source of metabolic activation. There are two versions of the assay, one using soft agar cloning to enumerate mutants (Clive, et al., 1979 and Turner et al., 1984) and one using liquid media and microwell plates (Cole, et al., 1986). Both versions of the assay are equally acceptable (Moore, et al., 2000).

IV. Principle of the Test Method

Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-}$ to $TK^{-/-}$ are resistant to the cytostatic effects of the pyrimidine analogue trifluorothymidine (TFT). 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, whereas normal cells, which contain the TK enzyme, are not.

Mouse lymphoma cells are grown and treated with test agents in suspension culture. Treatment should be done both with and without exogenous metabolic activation. Following treatment, cells are cultured to allow phenotypic expression prior to mutant selection. Cytotoxicity is measured and used to determine the appropriate dose range of the test chemical. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect the mutant cells, and in medium without the selective agent to determine cloning efficiency (viability). After a suitable incubation time, the colonies are counted. The mutant frequency is derived from the number of mutant colonies in the selective medium and the viability.

V. Description of the Method

A. Preparations

1. Cells

Because the assay was developed and characterized using the $TK^{+/-}$ -3.7.2C subline of L5178Y cells, it is important that the assay be conducted using $TK^{+/-}$ -3.7.2C cells (Mitchell, et al., 1997). It is advisable for all laboratories to karyotype the cells or paint the chromosome 11s to assure that there are two normal looking chromosome 11s and to identify any other irregularities. The karyotype for the $TK^{+/-}$-3.7.2.C cells has been published (Sawyer, et al., 1985, 1989). The modal chromosome number for the L5178Y/$TK^{+/-}$-3.7.2C cell line is 40. There is one metacentric chromosome (t12;13) that should be counted as one chromosome. Karyotyping and/or chromosome 11 painting should be performed when establishing a master stock. Cell cultures need to be monitored for doubling times. Normal doubling times are generally between 8 and 10 hr. Population doubling time should be checked when setting down master stocks. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. As a general guidance, if a laboratory is continually growing cells, the culture should be maintained for no longer than 3 months.

2. Media and Culture Conditions

Appropriate culture media and incubation conditions (culture vessels, temperature, CO_2 concentration and humidity) should be used. It is particularly important that culture conditions be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells. For the MLA it is also important that the culture conditions ensure optimal growth of the small colony TK mutants. Both Fischer's Medium for Leukemic Cells of Mice and RPMI 1640 media have been successfully used with the MLA.

The osmolalilty and pH of the medium should be confirmed to be in the physiological range (300 ± 20 mOsm and pH 7.0 ± 0.4). Each lot of horse serum should be tested for its ability to support optimal cell growth in suspension culture (low and high cell densities), high plating efficiency

and small colony mutant recovery (Turner et al, 1984).

3. Preparation of Cultures

Cells are propagated from stock cultures, seeded in culture medium and incubated at 37°C. Prior to use, the culture needs to be cleansed of pre-existing mutant cells. This is accomplished using methotrexate to select against TK-deficient cells. Thymidine, hypoxanthine and guanine are added to the culture to ensure optimal growth of the TK-competent cells (Turner et al., 1984).

4. Metabolic Activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β-naphthoflavone (Mitchell, et al., 1997). The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. The use of alternative metabolic activation systems such as primary hepatocytes (Brock, et al., 1987 and Oglesby, et al., 1989) should be justified.

5. Test Substance/Preparations

Liquid test substances may be added directly to the test system or diluted prior to treatment. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. It should be noted that problems may occur, particularly with suspension cultures, if the test substance precipitates either prior to addition to the cultures or during the treatment. It is generally best to avoid testing chemicals using conditions where they are either insoluble or become insoluble during the treatment. In some cases, it may be possible to use lower chemical concentrations and to increase the treatment time (without metabolic activation) so that the test can be performed under conditions where the chemical is soluble. Fresh chemical preparations should be employed unless stability data demonstrate the acceptability of storage.

The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in the MLA. For example, any suspected selective toxicity by the test substance for mutant and non-mutant cells should be investigated. If mutants and non-mutant cells are differentially sensitive to the test agent, the preexisting spontaneous mutants may be selectively increased in frequency during the treatment. The resultant increase in mutant frequency would be due to that selection of pre-existing spontaneous mutants rather than from the induction of new mutations. This possibility needs to be considered and investigated when the test chemical is structurally related to TFT.

B. Test Conditions

1. Solvent/vehicle

The solvent/vehicle should be chosen to maximize the solubility of the test agent. However, it should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed using a molecular sieve. Organic solvents that have been used with the MLA include DMSO, acetone and ethyl alcohol.

2. Exposure Conditions

Criteria to be considered when determining the highest test concentration include cytotoxicity, solubility in the test system, and changes in pH or osmolality. For relatively non-cytotoxic compounds the maximum concentration should be 5 mg/ml, 5 μl/ml or 0.01 M, whichever is lowest.

Relatively insoluble substances should be tested up to or beyond their limit of solubility under the culture conditions, although care should be exercised to minimize conditions that complicate quantification of results. Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and end or the treatment, as solubility can change during the course of exposure in the test system due to the presence of cells, S9, serum etc. Insolubility may be assessed using the naked eye.

In the MLA where the cells are grown in suspension, testing compounds in the precipitating range is problematical with respect to defining the exposure period. After the defined exposure period, the cells are normally pelleted by centrifugation and are then resuspended in fresh medium without the test compound. If a precipitate is present, the compound will be carried through to the later stages of the assay making control of exposure impossible. Therefore, for the MLA it is reasonable to use the lowest precipitating concentration as the highest concentration tested or the highest test concentration used in the data evaluation. This should help to minimize conditions that could complicate quantification of results.

3. Cytotoxicity

Cytotoxicity should be determined for each individual test and control culture. For the soft agar version of the MLA, this has generally been done using the relative total growth (RTG) which was originally defined by Clive and Spector (1975). This measure includes the relative growth in suspension during the expression time and the relative cloning efficiency at the time that mutants are selected. The microwell version of the assay was developed using the relative survival (RS) as the cytotoxicity measure. The RS is determined by the relative plating efficiency of each culture when plated immediately after the exposure period. The RTG and the RS are different measures of cytotoxicity and, although there is no real justification that one measure is superior to the other, it is important that the same measure of cytotoxicity be used for both versions of the assay. Because the RS is not normally measured in the soft agar version of the assay and the RTG is measured in both versions, it is recommended that the RTG be used as the standard measure of cytotoxicity. This cytotoxicity value is used both to determine the required

concentration range for an acceptable test and for establishing the highest concentration that is used for defining positive and negative responses (Moore, et al., 2000).

There are additional considerations in the calculation of the RTG between the two methods for the conduct of the MLA. In the agar method, the cells are exposed to the test chemical, the chemical is removed by centrifugation and resuspension in fresh medium. The first cell count takes place approximately 24 hrs after the initiation of the chemical exposure. On the first day following treatment, the cell density for each culture is readjusted, generally to 0.2 or 0.3×10^6 cells per ml of medium. Treated cultures with densities less than 0.2 or 0.3×10^6 cells per ml of medium are generally not adjusted in their density, and usually have sustained too much cytotoxicity to carry through the full experiment for mutant enumeration. For each treatment culture, the relative cell growth (compared to control) is calculated. On the second day following treatment, the cultures are again counted, adjusted in density and prepared to clone for mutant enumeration. The total two-day suspension growth of each culture is calculated and each treated culture is compared to the control. This value is referred to as the relative suspension growth (RSG). Cultures are cloned with and without selective medium to enumerate mutants and to calculate the mutant frequency (number of mutants per 10^6 cloneable cells). The relative plating efficiency for each culture is determined (relative to the negative control) and multiplied by the RSG to obtain a relative total growth (RTG).

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

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

To make the cytotoxicity measures obtained in the two versions equivalent, it is necessary for users of the microwell method to adjust their RS, RSG and RTG values to include the differential growth that can occur during treatment. This adjustment should be made by comparing the cell density in each treated culture with that of the negative control immediately following treatment. By comparing the growth of each treated culture relative to the control, it is possible to calculate a relative growth during treatment factor that can then be used to adjust the RS, RSG and RTG. As an example, if following the treatment period, the negative control had a cell density of 0.6×10^6 cell/ml and the treated culture had a density of 0.3×10^6 cell/ml, then the relative growth during treatment for that treated culture is 0.5 (or 50%). If the RS for that culture is determined to be 0.4 then the adjusted RS would be calculated as the RS × the relative growth during treatment or $0.4 \times 0.5=0.20$ (or 20%). The RSG would be adjusted in the same manner. The adjusted RTG would be obtained by multiplying the adjusted RSG by the relative plating efficiency at the time of mutant selection.

4. Dose Selection

The selection and spacing of doses is a critical factor in the proper conduct of the MLA. It is desirable to have more than one data point that can be used to confirm a positive or negative response. The assay may be conducted using either single cultures per dose point or multiple cultures per dose point. The strategy for determining the number of doses and the selection and spacing of doses can vary based on the toxicity range of the test material being evaluated and degree by which the chemical does or does not increase mutant frequency. It is not necessary to have a prescribed number of analyzable cultures when the chemical is clearly positive. When the chemical is not mutagenic or is only weakly mutagenic, generally, at least 4 analyzable doses are required when duplicate cultures are used and 8 analyzable doses are required when single cultures are used.

For toxic test materials, the highest dose level should induce an 80% reduction in RTG. Dose levels that induce more than a 90% reduction in RTG are usually excluded from the evaluation. However, as noted below, there are circumstances where the data points obtained at less than 10% RTG can be useful in the final evaluation.

While it is generally advisable to obtain data points covering the entire 100 to 10% RTG range, the validity of a test does not always depend upon attaining such a complete dose-response. When a test material induces large increases in mutant frequency, it is generally sufficient to provide data points anywhere within the 100-10% RTG range. For test materials that are not mutagenic or that induce only weak mutagenic responses, it is advisable to place emphasis on selecting doses that are expected to produce higher toxicity. This increases the probability of obtaining data points that can be used to make a definitive evaluation, that is, data points in the approximately 10-20% RTG range.

Therefore, it is recommended that laboratories attempt to achieve a maximum dose with RTG values between 10-20%. However, as already indicated, if a chemical clearly satisfies the criteria for a positive response the result will still be valid even if there is no test concentration resulting in 10-20% RTG.

When the mutant frequency is elevated at one or more doses above the background frequency (yet has not reached a level to be determined positive), interpretation of the test, and therefore its validity, will depend upon at least one dose that results in an RTG within the 10-20% range. This may only be achievable by conducting a repeat experiment in which the dose range is modified to increase the probability of attaining data points within the 10-20% RTG range.

There are some circumstances under which a chemical may be determined to be nonmutagenic when there is no culture showing an RTG value between 10-20 % RTG. 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 and there is at least one data point between 20 and 25% RTG. (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 between 10% and 1% RTG.

Significant increases in mutant frequencies seen only at RTG <10%, but with no evidence of mutagenicity at RTG >10%, do not constitute a positive result.

5. Controls

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

Positive controls should induce mostly small colony TK mutants. One appropriate positive control in the absence of S9 metabolic activation is methyl methanesulfonate. Appropriate positive controls to be used with S9 activation include cyclophosphamide (monohydrate), benzo(a)pyrene, and 3-methylcholanthrene. Positive control responses with and without S9 should be used for quality control measures and to demonstrate adequate detection of small colony mutants. Each laboratory must establish its own historical database for its positive and negative controls.

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

C. Procedure

1. Treatment with test substance

Cells, growing in log phase, should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time (generally 3-4 hrs is used). However, it may also be advisable (particularly for chemicals demonstrating insolubility) to extend the treatment time (without metabolic activation) to 24 hrs. Recently the International Conference on Harmonisation has recommended that all chemicals that are negative following the standard 3-4 hr treatment be evaluated (without metabolic activation) using a 24-hr treatment.

Either duplicate or single treated cultures may be used at each concentration tested. In either event, the number of concentrations used must be sufficient to provide confidence in the evaluation. Particularly, in situations where the chemical is negative or weakly positive, it may be advisable to use single treated cultures, and increase the number of different concentrations evaluated in a single experiment. Because of the importance of the negative controls, it is recommended that duplicate negative (solvent) control cultures be used.

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

2. Expression time and measurement of mutant frequency

At the end of the exposure period, cells are washed and cultured to allow for the expression of the mutant phenotype.

Each locus has a defined minimum time requirement to allow near optimal phenotypic

expression of newly induced mutants. For the TK locus that time is 2 days (Moore and Clive, 1982). Following the expression period, cells are grown in medium with and without selective agent for the determination of the numbers of mutants and cloning efficiency (used to calculate mutant frequency), respectively. This mutant selection can be accomplished using TFT selection (Moore-Brown et al., 1981) and either the soft agar or the microwell cloning method (Moore et al., 2000).

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

3. Mutant Colony Sizing

If the test substance is positive in the MLA, mutant colony sizing should be performed on at least one of the test cultures (generally the highest acceptable positive concentration) and on the negative and positive controls. Colony sizing can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events. If the test substance is negative, mutant colony sizing should be performed on the negative and positive controls. Colony sizing on the negative control is needed to demonstrate that large colonies are growing adequately. The test chemical cannot be determined to be negative if the positive control does not demonstrate the appropriate level of small mutant colony induction and detection.

VI. Data and Reporting

A. Treatment of results

Data should include cytotoxicity and plating efficiency determination, colony counts and mutant frequencies for the treated and control cultures. In the case of a positive response, mutant colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive controls.

The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail (Applegate, et al, 1990, Hozier et al., 1981, 1985 and Moore et al., 1985). Small and large colony mutants are distinguished by growth rate, and therefore they form colonies of differing size. Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies. The induction of small colony mutants has been associated with chemicals that induce chromosomal aberrations. Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

The RS (if determined), RTG and RSG should be given. Mutant frequency should be expressed

as number of mutant cells per number of surviving cells.

Individual culture data should be provided in tabular form that can be cross-referenced to the summary data. Additionally, all data should be summarized in tabular form.

While there is no requirement for verification of a clear positive response, a confirmatory experiment is often useful. Experiments that do not provide enough information to determine whether the chemical is positive or negative should be clarified by further testing preferably by modifying the test concentrations. Negative results using the short (3-4 hr) treatment should be confirmed by repeat testing using 24-hr treatment without metabolic activation. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

B. Evaluation and interpretation of results

There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency. The U.S. EPA MLA Gene-Tox Workgroup developed criteria for evaluating the published literature. These criteria for positive and negative (and other responses) can be used as guidance in interpreting data (Mitchell, et al., 1997). Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response. Biological relevance of the results should also be considered.

Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. In these situations, a chemical will give equivocal results in two or more very well conducted studies. In those situations, it is generally not useful to conduct a further study. Such chemicals are not negative and they should be considered to be borderline responses.

Positive results for the MLA indicate that the test substance induces mutations affecting the expression of the thymidine kinase gene in the cultured mammalian cells used. A positive concentration-related response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce mutations affecting the thymidine kinase gene in the mouse lymphoma cells.

It should be noted that positive results that may not be relevant to the *in vivo* situation may arise *in vitro* from changes in pH, osmolality or high levels of cytotoxicity (Brusick, 1986, Mitchell, et al., 1997 and Scott, et al., 1991).

VII. Test Report

The test report should include the following information:

A. Test substance:

- identification data and CAS no., if known;

- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance-including both the "neat" sample and the sample in the solvent/vehicle/medium. This should be done both prior to and at the end of the treatment period.

B. Solvent/Vehicle:

- justification for choice of vehicle/solvent;
- solubility and stability of the test substance in solvent/vehicle, if known.

C. Cells:

- type and source of cells
- karyotype of cells
- number of cell cultures;
- methods for maintenance of cell cultures;
- absence of mycoplasma.

D. Test conditions:

- rationale for selection of concentrations and number of cell cultures including e.g., cytotoxicity
- data and solubility limitations, if available;
- composition of media, $C0_2$ concentration;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature;
- incubation time;
- cell density during treatment;
- type and composition of metabolic activation system including acceptability criteria;
- positive and negative controls;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- selective agent(s);
- criteria for considering tests 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).

E. Results:

- signs of toxicity;
- signs of precipitation;
- data on pH and osmolality during the exposure to the test substance, if determined;
- colony sizing (for positive test chemicals) and for the negative and positive control

- dose-response relationship, where possible;
- statistical analyses, if any;
- concurrent negative (solvent/vehicle) and positive control data;
- historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations; number of tests upon which the historical controls are based;
- mutant frequency;
- Raw data, including cell culture counts and colony counts

F. Discussion of the results:

G. Conclusion:

VIII. References

The following references should be consulted for additional background information on this test guideline.

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