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Standard Guide for Performing the Mouse Lymphoma Assay for Mammalian Cell Mutagenicity[1]

This standard is issued under the fixed designation E 1280; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

This guide was developed at the request of ASTM Subcommittee E47.09 on Biomarkers in order to aid toxicologists, geneticists, biochemists, other researchers, and interested persons in the understanding, performance, and analysis of the mammalian cell mutagenicity test that uses the $TK^{+/-}$-3.7.2C strain of L5178Y mouse lymphoma cells. In this rapidly changing area of toxicology, it is not intended for this guide to replace, alter, or diminish the usefulness of presently available protocols and procedures.

1. Scope

1.1 The purpose and scope of this guide is to present background material and to establish criteria by which protocols and procedures for conducting the L5178Y/$TK^{+/-}$-3.7.2C mouse lymphoma mutagenicity assay (commonly referred to as the mouse lymphoma assay, (MLA)) can be properly understood and evaluated. This guide is also intended to aid researchers and others to gain a better understanding of the critical elements involved with mammalian cell mutagenicity testing. More specifically, this guide is intended to provide for researchers the accomplishment of the following goals:

1.1.1 Provide an understanding of the critical procedures (steps) in the performance of this mammalian cell mutagenicity test.

1.1.2 Provide generalized criteria by which researchers can evaluate if they are properly performing, utilizing, and interpreting this assay.

1.1.3 Provide criteria by which individuals responsible for evaluating MLA data can determine if the experiments have been properly performed and interpreted.

1.1.4 Provide a basis from which new procedures and developments in testing procedures can be evaluated.

1.1.5 Provide an understanding of the types of genetic damage (that is, gene and chromosome mutation) that may be detected in this mammalian cell mutagenicity test.

2. Terminology

2.1 *Definitions:*

2.1.1 *clastogen*—any agent that is capable of inducing chromosome breaks.

2.1.2 *gene mutation*—any heritable change whose physical extent is restricted to the limits of a single gene.

2.1.3 *mutagen*—any physical or chemical agent capable of inducing a mutation.

2.1.4 *mutation*—any heritable change in the genetic material, not caused by genetic segregation or genetic recombination, and that is transmitted to daughter cells.

2.2 *Definitions of Terms Specific to This Standard:*

2.2.1 *chromosome mutation*—a mutation resulting from a structural change to a chromosome involving the gain, loss, or relocation of chromosome segments. Chromosome mutations can be either intrachromosomal or interchromosomal.

2.2.2 *relative suspension growth (RSG)*—used to measure the cytotoxicity of a given treatment based on the growth of cells in suspension culture relative to the untreated or solvent control(s). RSG is calculated according to the method of Clive and Spector **(1)**.[2]

2.2.3 *relative total growth (RTG)*—used as a means to measure the relative toxicity to cells (survival) following treatment in the mouse lymphoma assay. RTG is calculated according to the method of Clive and Spector **(1)** and includes RSG as well as the ability to form colonies in the clonal phase of the assay.

2.3 *Symbols:*

2.3.1 *BrUdR*—5-bromo-2′-deoxyuridine.

2.3.2 *BUdR*—bromouracil deoxyriboside.

2.3.3 *CAS*—chemical abstract service.

2.3.4 *DMSO*—dimethylsulfoxide.

2.3.5 *MLA*—mouse lymphoma assay.

[1] This guide is under the jurisdiction of Committee F04on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this guide.

2.3.6 *NADP*—nicotinamide-adenine dinucleotide phosphate.

2.3.7 *TFT*—trifluorothymidine.

2.3.8 *THMG*—thymidine + hypoxanthine + methotrexate + glycine.

2.3.9 *VC*—viable count(s).

3. Significance and Use

3.1 This guide is limited to procedures used solely for the testing of substances to determine their mutagenicity and does not apply to other methods and uses such as exploring mechanisms of mutation.

3.2 Recent evidence suggests that this assay measures a dual genetic end point; therefore, some discussion of the relationships between mammalian cell mutagenicity testing results and the results observed both in pure gene mutational assays and in cytogenetic assays is necessary. However, it is not the intent of this guide to discuss other relationships between this mammalian cell mutagenicity testing results and the results observed in other tests for mutagenicity and carcinogenicity.

4. Test Materials

4.1 *Media*—Fischer—**(2)** successfully adapted L5178Y mouse leukemic cells to growth in suspension culture using F10 (Gibco H-11) medium. In developing and validating the L5178Y mouse lymphoma assay, Clive and associates **(1)** routinely used Fischer's medium; however, other laboratories have recently validated the assay with RPMI 1640 medium **(3-5)**. Either medium can be used; however, it is important to note several differences between them. The most important of these is the large difference in phosphate concentration, a factor which can affect the stringency of trifluorothymidine (TFT) selection in RPMI medium **(6)** if proper precautions concerning heat inactivation and quality of horse serum are not taken **(7)**; (see 4.1.4.1). Secondly, the effective concentrations of cleansing medium components is dependent on the type of base medium used (see 4.1.4.2). It is recommended that critical components (for example, horse serum) be heat-inactivated either separately or after combination. Fischer's medium is photosensitive in liquid formulations!

4.1.1 *Base Medium*—A base medium is generally prepared from powdered formulation or is purchased as a 10× or 1× liquid. Some laboratories prepare 2× medium which can be used for a variety of media preparations. Pluronic F68[3] must be added to the base medium to facilitate growth in suspension culture. Other supplements usually include antibiotics, sodium pyruvate, and occasionally, glutamine. Refer to references in 4.1 for suggested concentrations.

4.1.2 *Growth Medium*—Growth medium is prepared by supplementing the base medium with horse serum, usually 10 % by volume.

4.1.3 *Cloning Medium*—Cloning medium is growth medium further supplemented with agar (Noble, purified, or Baltimore Biological Laboratories (BBL); see Ref. **(8)**) and often with additional serum. Each investigator should determine serum and agar concentrations that yield the best cloning conditions in their laboratory. See references in 4.1 for agar and serum concentrations as they vary between laboratories. Serum concentration is often adjusted to 20 % in the cloning medium since this concentration has been reported to provide the highest cloning efficiency for L5178Y cells **(9)**; however, this optimum may vary among lots of horse serum and among laboratories.

4.1.4 *Selective Media*—There are two types of selective media routinely used in the MLA: cloning medium supplemented with TFT to permit quantitation and characterization of $TK^{-/-}$ mutants; and THMG cleansing medium which keeps the spontaneous $TK^{-/-}$ mutant frequency at a minimum, thereby optimizing the assay sensitivity.

4.1.4.1 *TFT Selection*—Cloning medium supplemented with TFT is used to arrest growth of $TK^{+/-}$ cells and to allow clonal growth of $TK^{-/-}$ cells. The optimal concentration of TFT may vary among laboratories, but is usually in the range of 1 to 5 µg/ml. Those laboratories utilizing RPMI 1640 medium may find it necessary to use a TFT concentration at the higher end of this range. Each laboratory should establish the efficacy of their TFT selection by appropriate means. Differential lots of horse serum vary in their ability to inactivate TFT, possibly resulting from varying amounts of the enzyme thymidine phosphorylase. This enzyme, in the presence of inorganic phosphate, converts TFT to an inactive form. The approximately sixfold higher level of inorganic phosphate present in RPMI 1640 medium (relative to Fischer's medium) drives this inactivation more rapidly in RPMI-based cloning medium if the serum is improperly heat inactivated, thereby critically decreasing TFT-selection stringency in the mutant selection plates. This can be overridden by a combination of increased TFT concentration, extra attention to the proper heat inactivation of the horse serum (that is, ensure that the serum reaches 56°C prior to initiating the 30 min incubation; Mayo, unpublished data) **(2, 11)**, and stringent screening of serum lots prior to routine use in the assay.

NOTE 1—Historically, 5-bromo-2′-deoxyuridine (BUdR; BrUdR) has been utilized with this assay to select for $TK^{-/-}$ cells. TFT has been shown to be a more effective selective agent, and the use of BUdR is discouraged **(10)**.

4.1.4.2 *THMG Cleansing*—Cleansing medium (growth medium supplemented with THMG) is one method used to rid the stock culture of spontaneously accumulated $TK^{-/-}$ mutants. It is composed of: methotrexate (M), to block folate-dependent thymidylate synthase production of thymidine monophosphate (TMP), thus forcing the cells into dependency on the TK salvage pathway of TMP synthesis; thymidine (T) and hypoxanthine (H), to bypass the folate block in TK-competent cells; and glycine (G) as a methyl group source. In $TK^{-/-}$ mutant cells, the exogenous thymidine cannot be phoshorylated, and these cells die from TMP deficiency. Following 24-h growth in the cleansing medium, the stock culture is centrifuged and the cells are washed free of unbound methotrexate and resuspended in growth medium supplemented with THG (that is, THMG without methotrexate) for 1 to 3 days. This permits the

[3] The sole source of supply of the apparatus known to the committee at this time is BASF Wyandotte Corp., Wyandotte, MI 48192. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

cells to fully recover from the remaining bound methotrexate and resume synthesis of TMP and purines by the folate-dependent pathways. Cells should be allowed to totally recover from the metabolic stress of the cleansing procedure (about 2 to 3 days) before being used in a test.

4.1.4.3 While it has been suggested that the cleansing procedure be performed on a weekly basis, some laboratories may find a less frequent cleansing schedule acceptable, providing a low background mutation frequency is maintained. Other alternatives include: freezing populations of freshly cleansed cells and thawing them a few days prior to use; using cultures grown for a very low inoculum (ca. 600 cells/culture; however, this method suffers from potential genetic drift problems which could alter this well-characterized cell line); or maintaining an uncleansed population of cells and cleansing a portion of these cells prior to use. In these cases the exposure of $TK^{+/-}$ cells to methotrexate, which, in the absence of THG is known to induce mutations, can be reduced to a minimum. For specific concentrations of the ingredients and cell populations used in the cleansing step, refer to references in 4.1. It is important to note for those laboratories utilizing RPMI 1640 medium, that slightly higher concentrations of THMG and THG are required, as noted in the literature.

4.1.5 *Quality Control of Media*—The quality of culture media is a common cause of problems with the MLA. A number of factors are known to contribute to variations in medium quality, the principal ones being water quality and exposure of liquid Fischer's medium to excessive light. Another identified source of assay problems is the lot and source of agar **(8)** and the problem of the use of a dirty autoclave to sterilize the agar. Serum requires particular precautions with RPMI medium **(7, 11)**; see 4.1.4.1. For these reasons, rigorous methods for media quality control should be established for each laboratory to address the ability to support: (*1*) suspension growth of both low (≤1000 cells/mL) and high ($>1 \times 10^6$ cells/mL) cell inocula, (*2*) high cloning efficiencies under nonselective conditions, (*3*) adequate recoveries of small and large colony $TK^{-/-}$ mutants, and (*4*) appropriate diameters of nonmutant and both classes of mutant colonies. Each of these quantities should be consistent with published literature values.

4.2 *Metabolic Activation System*—The metabolic activation system may take the form of either whole cells (for example, cocultivated rat hepatocytes **(12 and 13)**) or cell homogenates (for example, Aroclor-1254–induced rat liver S9 **(14)**; Aroclor-1254–induced hamster or mouse S9 **(15)**).

4.2.1 *Sources*—Preparations designed to provide metabolic activation may be prepared from a variety of sources depending on the needs of a particular assay. Factors which may vary include, for example, species, sex, tissue, age, method of induction, and method of preparation.

4.2.2 *Cofactor Mixes for Enzyme Preparations*—should be shown to support enzyme activity, as measured either directly or by a biological effect. Commonly used cofactors include NADP in conjunction with either sodium isocitrate or glucose-6-phosphate **(3, 5, 14-16)**.

4.2.3 *Metabolic Activities*—The metabolic activation system to be used should be capable of converting appropriate known promutagens to mutagens while causing little or no toxicity or mutagenicity to the mouse lymphoma cells in the solvent control culture(s).

5. Test Method

5.1 *Test Principle*—The mouse lymphoma assay utilizes a strain ($TK^{+/-}$-3.7.2C clonal line) of L5178Y mouse lymphoma cells that has been made heterozygous at the TK locus **(17)**. These cells contain the TK enzyme and are sensitive to the cytostatic and cytotoxic effects of appropriate concentrations of TFT **(10)**. Forward mutations to the single functional TK gene can result in the loss of TK activity and thus the acquisition of TFT-resistance. These mutant cells can be quantitated after an appropriate expression period by cloning in a soft agar medium supplemented with the selective agent, TFT **(10, 18)**. A number of protocols have been described **(1, 14-19)**. The assay has been adapted to detect a wide variety of mutagens including those requiring exogenous metabolic activation.

5.2 *Description of Test System:*

5.2.1 *Cell Line*—The MLA uses the $TK^{+/-}$-3.7.2C heterozygote of L5178Y mouse lymphoma cells **(17)**. This cell line has been cytogenetically characterized by banded karyotype at the 230 to 300-band level of resolution **(20 and 21)**. The chromosome 11 homologs, the known location of the TK gene in the mouse **(22)**, have been shown to possess a centromeric heteromorphism that distinguishes the chromosomes 11*a* and 11*b* (small and large centromeres, respectively) **(23)**. Through banded karyotype analysis of a large number of $TK^{-/-}$ mutants, this property has allowed the provisional mapping of the single functional TK gene to the terminal two-band region of chromosome 11*b* in this cell line **(23)**. It is recommended that these cells be obtained from D. Clive in order to minimize interlaboratory variability.

5.2.2 *Mutational End points*—This cell line forms two classes of TK-deficient (TFT-resistant) mutants based on the criterion of colony size in soft agar cloning medium supplemented with a selective concentration of TFT. Both large and small TFT-resistant colonies are totally and heritably TK-deficient by direct enzyme assay **(6, 14, 18, 24, 25)**. Further, the majority of small colony TK-deficient ($\sigma TK^{-/-}$) mutants possess chromosome 11*b* abnormalities ranging from two band insertions or deletions up to whole chromosome translocations, whereas most large colony TK-deficient ($\lambda TK^{-/-}$) mutants appear karyotypically indistinguishable from the parental $TK^{+/-}$-3.7.2C cells at this same level of resolution **(25-27)**. Thus, the mouse lymphoma assay appears to detect genetic damage ranging from single gene alterations to viable chromosomal damage affecting the TK locus.

5.2.3 *Storage*—These cells should be properly stored in liquid nitrogen according to published procedures **(1, 3)**.

5.2.4 *Integrity of the Test System*—There are a number of parameters that can be monitored to assess the integrity of the test system. Each laboratory should establish quality control criteria, consistent with the published literature, in order to establish optimum quality of such variables as: water, media components, horse serum, incubator conditions, TFT, agar, plastic or glass cell containers, cell and colony counters, and so forth. Monitoring the following factors is especially important for the establishment of historical data and ranges in a particular laboratory.

5.2.4.1 *Cell Growth Rates*—Each laboratory should establish cell growth conditions so that stock cells are maintained in exponential growth with a population doubling time of 10 ± 2 h. Special attention should be given to the growth rate and general appearance (including microscopic examination) of cell populations following the THMG cleansing procedure. A slight reduction in the growth rate at this time is not unusual but major deviations from the normal range indicate suboptimal health of the cells, a problem with the cleansing medium, or possible mycoplasma contamination.

5.2.4.2 *Plating (Cloning) Efficiencies*—Plating efficiency may be a good indicator of the health and vigor of the cells at the end of the expression period, but in general it probably is a better indicator of how well the cloning process and subsequent incubation were controlled. Ideally, absolute plating efficiencies (PE) of the solvent control cultures should be at least 75 %, but lower efficiencies are acceptable providing the results of the experiment are not compromised. Some factors that may reduce cloning efficiency are: (*1*) temperature of the cloning medium (CM); (*2*) viscosity of CM; (*3*) pH of CM; (*4*) improper disaggregation of cell clumps prior to addition to CM; (*5*) poor control of pH and temperature during the incubation period; (*6*) insufficient duration of the incubation period; (*7*) poor quality of medium components, especially serum and agar **(8)**; and (*8*) overgrowth of cells in suspension culture prior to cloning. Assays with negative results would be considered acceptable if the PE of the solvent control cultures are at least 60 %. Assays with strong dose dependent responses may be acceptable with lower cloning efficiencies (that is, 50–60 %), but should be judged on a case-by-case basis; a repeat assay is strongly recommended in such instances. An experiment with a solvent control PE below 50 % is unacceptable. An experiment with a solvent control PE exceeding 100 % is acceptable providing it does not jeopardize the proper assessment of mutagenicity. Experiments consistently producing PE's exceeding 100 % may indicate technical error associated with cell or colony counting or the cloning procedure, or both. A solvent control PE exceeding 150 % is unacceptable.

5.2.4.3 *Spontaneous Mutant Frequency*—The spontaneous (background) mutant frequency may vary considerably among laboratories and even within the same laboratory. Each laboratory should use not only published ranges of response but also its own historical data base with self-imposed limits for determining an acceptable spontaneous mutant frequency. The presence of the metabolic activation system may increase, decrease, or leave unaffected the spontaneous mutant frequency. $TK^{+/-}$ cells require appropriate periodic purging of $TK^{-/-}$ cells (see 4.1.4.2) that are accumulating spontaneously in the stock population. Such cleansing on a regular basis will decrease the background mutant frequency and is required to prevent inflated background mutant frequencies.

5.2.4.4 *Positive and Negative Controls*—Presently, there are no mandatory reference substances for use as concurrent positive or negative controls, or both. However, negative controls are usually cultures treated with the solvent used to solubilize and dilute the test compound. Only concentrations of solvent that have no effect on cell growth, cell survival, and mutant frequency should be used. Solvents commonly used in the MLA are dimethylsulfoxide (DMSO), saline, water, serum-free medium, ethanol, and, less frequently, acetone. These solvents have no detectable effect when applied at concentrations of 1 % or less. If higher concentrations or alternate solvents are used, sufficient testing should be performed to establish acceptable limits. If a substance is being tested under metabolic activation conditions, the negative controls should also be run under those same conditions. Select positive control compounds for the purpose of detecting any compromise of integrity of the test system. If the test compound is being assessed in the presence and absence of an exogenous metabolic activation system, then a direct-acting mutagen and one requiring metabolic activation must be included in the assay as positive controls. A data base for each positive control compound sufficient to establish upper and lower response limits for the dose(s) used should be established prior to using them to evaluate an assay's acceptability. Assays with negative results having positive control responses below the acceptable range must be repeated; negative assays having positive control responses above the acceptable range should be carefully evaluated before acceptance.

5.2.5 *Metabolic Activation*—Test compounds should be tested both in the presence and absence of a suitable exogenous mammalian-metabolic activation system to permit the detection of mutagenic metabolites. (An exception might be made for testing under only one condition of metabolic activation if the test compound is clearly positive under that condition.) The metabolic activation system usually used in the MLA is Aroclor-induced rat liver S9-plus cofactors. However, the selection of the activating system should be flexible, allowing the investigator to select the system that optimizes the metabolism of the test compound. Therefore, the activation system should be chosen on the basis of its potential for activating the test compound, and should be monitored for its ability to induce an acceptable response in the positive controls.

5.2.6 *General Testing Procedure*—Although there are a variety of procedures that can or should be used, the following provides a description of the test as generally performed.

NOTE 2—These cells are adapted to suspension growth and should be grown with adequate agitation (to maintain normal cell doubling times) except after pouring plates for the cloning phase.

5.2.6.1 *Solubility Determination*—Prior to the first MLA experiment, the compound's solubility in solvents of choice should be determined. For DMSO and certain other nonaqueous solvents (5.2.4.4), a target stock concentration is 100 times the highest concentration expected to be tested to provide a final solvent concentration no greater than 1 %; lower multiples of the highest expected test concentration are sufficient for saline, water (with due regard for nutrient dilution or hypotonicity), or serum-free medium. In instances of low solubility relative to cytotoxicity, a 1.0× stock solution can be prepared in serum-free medium; this can be added to cells pelletted from their growth medium in their individual tubes. In addition, certain test conditions may require the extraction of insoluble materials (that is, medical devices, or biomaterials) with subsequent testing of leachates. However, the solvent used for leaching the materials should adhere to the constraints mentioned above for solvent type and concentration.

5.2.6.2 *Toxicity (Range-Finding) Test*—A preliminary range-finding test is usually conducted at concentrations spanning several orders of magnitude, both in the presence and absence of an appropriate exogenous mammalian-metabolic activation system. Toxicity in the range-finding test is usually expressed as RSG or RTG. Final testing is conducted in the presence and absence of mammalian metabolic activation at several concentrations based on the results of the range-finding test. These concentrations should include one that will produce a RTG approaching 10 % that of solvent controls, where possible, and necessary to demonstrate mutagenicity. The highest concentration used will depend on the solubility and toxicity of the test compound but generally should not exceed 5 to 10 mg/mL. Selection of the maximum treatment level should be made on a compound-by-compound basis with special attention to factors such as solubility, toxicity, osmolarity, trace contaminants, and pH of the test material, each of which might contribute to the induction of a mutagenic effect **(28-30)**.

5.2.6.3 *Treatment*—Sufficient numbers of cells should be treated to permit adequate statistical conclusions. Their treatment should be for long enough time and to high enough concentrations of test compound to permit adequate assessment of mutagenic activity. Treatment of 6×10^6 cells with the appropriate concentrations of test compound or substance is typically for 4 h at 37°C. Each series of treatments (that is, with or without exogenous metabolic activation) consists of at least duplicate solvent controls, appropriate positive control(s), and culture(s) at each dose level. It is possible to obtain a false positive result if the spontaneous mutant frequency is too low for the number of cells treated. For example, a typical nonmutagenized culture containing 6×10^6 cells could, when treated to 10 % RSG, contain as few as 6×10^5 $TK^{+/-}$ cells if cell killing were immediate. This would leave in such a treated culture as few as 12–60 $TK^{-/-}$ mutants (corresponding to spontaneous mutant frequencies of $(20–100) \times 10^{-6}$). A positive result could occasionally arise at the low end of this range solely from stochastic effects. For this reason, sufficient cells should be treated in each culture to avoid such stochastic effects.

NOTE 3—Many laboratories prefer to dose in duplicate or triplicate. Following treatment, the cells are washed free of compound or substance by repeated centrifugations and resuspended in fresh culture medium at a cell concentration, usually in the range of $2:3 \times 10^5$ cells/mL, that is compatible with continued exponential growth.

5.2.6.4 *Expression*—Each treated and control culture is typically maintained with sufficient agitation for around 2 days at 37°C with daily cell counts and dilutions to maintain exponential growth and to allow expression of any induced $TK^{-/-}$ mutations **(31)**. Expression periods of shorter or longer duration may be optimal for certain compounds and should be determined if optimal positive responses are required. Expression times significantly longer than 2 days may result in a negative response even for mutagenic agents **(14, 31)**.

5.2.6.5 *Cloning*—Following expression of induced $TK^{-/-}$ mutants, appropriately selected cultures are cloned in soft agar cloning medium to determine the number of viable cells (VC plates; 300 to 600 cells per culture) and the number of mutants in the presence of stringent selective concentrations of TFT (TFT plates; $(1.5–3.0) \times 10^6$ cells per culture). Cloned cells are incubated at 5 % CO_2, 37°C for 9 to 12 days to allow colonial growth; the optimal conditions should be determined for each laboratory.

5.2.6.6 *Scoring and Sizing Colonies*—After incubation, each set of VC and TFT plates is scored for number of colonies. In addition, the colonies on the TFT plates can be electronically sized in order to determine the number of large and small colony mutants.

6. Test Data

6.1 *Treatment of Results:*

6.1.1 *Total Growth*—Most mammalian cell mutagenicity assays determine survival by cloning shortly after treatment. One difficulty with this is that, for a few hours following treatment by some compounds, some cells cannot survive when sparsely distributed (as in a determination of plating efficiency) but can survive under crowded tissue culture conditions where, for example, metabolic cooperation effects can occur; this results in a spuriously low estimation of survival. Since mutants and mutant progenitors are carried in mass culture, it makes more sense to measure viability under the same conditions. To measure the cytotoxic effect of a given treatment, a quantity called total growth is measured in the MLA. This amounts to determining, for each cell entering into the treatment, how many progeny are produced, relative to solvent traded controls, by the time that the cells have recovered from the treatment. Cells unable to divide a minimum of approximately 10 times will not form detectable colonies, resulting in a decrease in total growth. In addition, viable cells with transiently suppressed growth rate will have a somewhat decreased total growth due to slower growth in suspension culture; these later may form detectable colonies. Thus, total growth can serve as a sensitive indicator of relevant chemical-biological interactions that plating efficiency determinations would miss.

6.1.2 *Gathering Data*—All data should be collected in an orderly fashion to facilitate a rapid and accurate assessment of mutagenicity. Data should be well documented and should include all pertinent information regarding (*1*) maintenance of cell stock cultures; (*2*) preparation of all cell cultures for testing; (*3*) cell counts and post-exposure manipulations (days one and two); (*4*) suspension growth; (*5*) individual and mean colony counts; (*6*) cloning efficiencies; (*7*) total survivals (or total growths, both absolute and relative); (*8*) mutant frequencies; (*9*) relative proportions of large and small mutant colonies (optional but desirable); and (*10*) any other preferred mutation indices or statistical procedure applied, or both. Depending on the scope of the operation, a laboratory may automate certain aspects of the assay. For example, the use of a Coulter particle counter and electronic colony counter interfaced with a data acquisition system adds to the timeliness and accuracy in the collection and calculation of data.

6.1.2.1 *Daily Growths*—Monitoring daily growth verifies that the solvent control cells yield normal growth rates. In treated cultures, the presence and extent of cytotoxicity is measured and compared to concurrent negative controls. While many laboratories utilize a Coulter counter in assessing daily

cell counts, others prefer a light microscope and hemacytometer for cell counting; although this latter procedure is more labor intensive and time consuming, when performed with trypan blue stain a crude measure of cell viability can be obtained.

6.1.2.2 *Counting VC Plates*—After a suitable incubation period (see 5.2.6.5), the petri dishes are examined for artifactual particles and colonies are counted. This can best be done with an electronic colony counter for reasons of speed and possible direct interface with a computer. With proper calibration, electronic colony counter compares favorably with manual counting without the disadvantage of scorer fatigue. Generally, VC plates do not require the sizing of colonies, which are mostly uniformly large. The VC plates measure cloning efficiency (CE) that should fall within certain limits (see 5.2.6.5). The CE values may be responsible for the rejection of one or more treatments within an experiment or even the entire experiment; therefore, the proper counting and reporting of VC plate counts is necessary.

6.1.2.3 *Counting Mutant Colonies*—There are similar advantages to using an electronic colony counter for counting mutant colonies. Further, important information on the type of genetic damage being induced can often be obtained by sizing the mutant colonies in addition to simply counting them. It is important to determine the limits of the particular colony counter in counting the very smallest of the small colonies **(25)**. Most electronic counters do not count all colonies, and no attempt is made to adjust for the varying number (by chemical and dose) of very small colonies that occur on a plate.

6.1.2.4 *Sizing Mutant Colonies*—Two classes of TK-deficient (TFT-resistant) mutants, based on colony size and relating to the type of genetic damage induced, have been identified in the MLA (see 5.2.2). Certain compounds such as methyl methanesulfonate and hycanthone produce predominantly small colony mutants; a few compounds, such as ethyl methanesulfonate, produce predominantly large colony mutants. However, most compounds produce a bimodal population of TK-deficient mutant colonies **(14, 32)**. For these reasons some laboratories may choose to quantitate the frequencies of each of these two types of mutant colonies. It is important that all colonies be scored in the assessment for mutagenicity although, at present, separate enumeration of small- and large-colony mutants is not universal.

6.1.3 *Entering Data*—Daily cell concentration determinations and individual plate counts must be recorded in a permanent manner. Total growth and mutant frequency should be calculated for each treated and cloned culture, and concurrent control data should be added to the historical control data base.

6.1.4 *Plotting Data (optional)*—When test data are clearly negative, graphical representation does not usually provide additional information and ordinarily need not be done (except for the purpose of accumulating historical data, especially in cases in which data acquisition and processing are fully automated). Visualization of data in graphical form often aids in their interpretation, so that manual or computer-directed plotting of results is a useful step.

6.2 *Statistical Evaluation*—Many test results are clearly negative or positive, and statistical analysis is not needed to provide this answer. What is needed by investigators using the test is a consistent and acceptable method for analyzing experiments, especially those with questionable results. Many laboratories use the “twofold” or “modified twofold” rule, although this approach does not formally take into account the differences and variability in spontaneous backgrounds among various laboratories. Since most dose response curves in the MLA are nonlinear, analysis of variance based on linear models is inappropriate. Where there is doubt, investigators are encouraged to incorporate the use of an established statistical method into data analyses providing, of course, that the method is appropriate for MLA data and the appropriate statistical assumptions are met.

6.2.1 The majority of test data in the literature has not been evaluated using formal statistics. In general, decision of positive and negative are made on an ad hoc basis. A number of reports in the literature rely either on statistical methods or on the twofold rule. This rule states that if a doubling of mutant frequency over an appropriate background **(14)** is seen (for example, mutant frequencies in excess of 120×10^{-6} for a concurrent mean background mutant frequency of 60×10^{-6}), the chemical is considered mutagenic. The number of doses or replicates is not a factor in this evaluation. The mutagenic and cytotoxic responses should be reproducible and preferably dose related. Generally a mutagenic response is more convincing if more than one dose is positive by this criterion.

6.2.2 Recently, a number of statistical procedures have been proposed for the MLA **(4, 14, 33-36)**, but there is no consensus as to which particular procedure(s) should be used for data evaluation. It is clear, however, that regardless of the statistical evaluation procedure used, the data evaluated must meet a number of criteria, such as: it must be derived from a defined protocol covering a range of doses both with and without metabolic activation, the results should be reproducible, and the anticipated carcinogenicity or noncarcinogenicity of the chemical must not be a consideration in the evaluation.

6.3 *Interpretation of Results*—Before results can be interpreted, a number of experiments must be performed in each laboratory so that quality control criteria can be defined. These quality control criteria may include limits on the ranges of suspension growth, mutant frequency, and cloning efficiency for the solvent and positive controls, as well as toxicity limits for the treated cultures. While general guidelines can be obtained from the literature, these quality control criteria should be determined for each individual laboratory and should be consistent with literature values. Experiments with known mutagens/carcinogens and nonmutagens/noncarcinogens should be performed in each laboratory to establish response criteria.

6.3.1 After quality control criteria have been applied to a set of data, the results can be evaluated. The methods used must be consistent from experiment to experiment and have a justifiable

scientific basis. The reproducibility of a particular response elicits confidence when analyzing results, especially for compounds manifesting a weak mutagenic response. Such reproducibility may be demonstrated by performing replicate experiments and may be further substantiated by treating replicate cultures. For clearly positive responses, replicate experiments can often be obtained by cloning the appropriate portions of the dose-ranging experiment and comparing those limited results with the results of the subsequent mutagenicity experiment.

6.3.2 An appropriate statistical approach may be helpful in interpreting certain results. Compounds, mixtures, or preparations that produce mutagenic responses that meet the statistical criteria for a positive response may be considered to be positive under the particular experimental conditions. Statistical criteria should include an analysis of individual doses as well as an analysis of the trend of the dose response curve. Experiments that are positive only after precipitation or under conditions of high osmolarity, low pH, or other extreme condition should be noted. If results show a response of marginal significance at only one concentration, efforts should be made in a repeat trial to select doses around that concentration.

6.3.3 *Requirements for Declaring a Chemical or Test Mixture Mutagenic*—After the experiment has been subjected to the quality control criteria, the data over an appropriate relative total growth range **(4, 14, 37)** should be evaluated for doses that are positive and for trends in the dose response. Statistical methods or a twofold rule may be applied to the data; in all instances, the appropriateness of the method should be considered. If, after applying these principles to the test data, the compound cannot be evaluated as positive, then an evaluation of questionable or negative mutagenicity should be applied to the result.

6.3.4 *Requirements for Declaring a Chemical or Test Mixture Nonmutagenic*—An experiment can be called negative only if the quality control criteria set up by that laboratory are strictly met. In particular, positive controls results must be unambiguous (5.2.4.4). In addition, minimum upper dose criteria must be met, based either on concentration or cytotoxicity. Test concentrations should be spaced sufficiently close to preclude missing mutagenic activity on a steep survival curve. Only when these minimal conditions are met can a compound be considered to be nonmutagenic under the experimental conditions to which it was subjected. Experiments that meet the quality control criteria but cannot be evaluated as positive or negative should be considered questionable.

7. Test Report

7.1 *General*—The test report, whether for submission to a journal or other forums for publication in the open literature or for submission to a regulatory agency, should be as complete as possible and should contain: (*1*) enough detail to insure the reviewer that the test was performed according to accepted procedures, and (*2*) sufficient data to allow verification of the results. Certain critical elements that should be considered by the investigator when preparing a report are set forth below. Where the needs of the two types of report may differ, this is noted.

7.2 *Title*—The title should be as complete as possible and should accurately reflect the content of the report.

7.3 *Test Agent*—The name used to identify the test agent should be as complete as possible and may include the Chemical Abstract Service, (CAS) registry number, if available, and any commonly used synonyms. A representation of the molecular structure may be given. The source and, if appropriate, lot number of the test agent should be supplied. Complete information as to purity should be given; if analysis for purity has been performed, the method used may be referenced. Analyses not performed by the author of the report may also be referenced. A distinction should be made between technical and reagent grade substances, if appropriate. Test agents used as they were received from the supplier with no further attempt at analysis or chemical identification should be noted. Mixtures should be identified, and the percent composition of components should be given, if known or readily available. The solvent used should be identified, and the stability of the test agent both in the solvent and under laboratory storage conditions should be given, if known. Any special procedures used to enhance stability (for example, storage in the cold or dark) should be noted. The physical properties of the test agent may be described, and the physical state tested may be given. The interval between receipt of the chemical, preparation of stock solutions, and use in the assay should be stated, and storage conditions during this time should be noted. Any other information about the test agent which the author feels would be relevant should be presented. Where the information is not available or is deemed inappropriate by the author, this should be noted, especially for reports that may be presented to a regulatory agency for review and evaluation.

7.4 *Test System*—The test system should be well documented, and the cell source identified. Procedures used in maintaining the efficacy of the test system, that is, screening for mycoplasma contamination, mutant cleansing (THMG), enzyme analysis, karyotyping, etc should be clearly described in the standard operating procedures. Any additional procedures or modifications to the test system must be clearly outlined and explained.

7.5 *Procedures*—When writing for submission to a journal or other open literature publication, the author should bear in mind that the aim of this section is to permit repetition of the test conditions and to insure reproducibility of the reported results. Methods should be reported as concisely as possible with references to well-established procedures; such citations should be to seminal papers, not to secondary sources. In preparing a report for agency submission, the author should bear in mind that the aim of this section is verification that the test was performed according to acceptable standards; any information relevant to this determination should be presented. In particular, the procedures used should be detailed as completely as possible. When a published guideline (for example, Organization for Economic Cooperation and Development (OECD) or Environmental Protection Agency (EPA)) is being followed, this should be clearly stated. Modifications to the guidelines, to the stated protocol, or to any relevant laboratory standard operating procedures (SOP) should be documented, and the reasons for deviations should be clearly

stated. Relevant SOPs (for example, for preparation of the metabolic activation system) should be retained by the author and a copy submitted upon request.

7.6 *Experimental Design*—This section should include the number of concentrations tested, the rationale for dose selection, toxicity information, and information on factors that may affect the results. The latter may include, but is not limited to, solvent, pH deviations, duration of treatment, composition of the media, artificial lighting conditions, and length of incubation of mutant and viable count plates. The number of replicates, both within an experiment and of the entire experiment, should be indicated. Positive and negative control data should be presented; for submission to a regulatory agency the historical range of mutant frequencies should be given. Details of the metabolic activation system used should be presented. This may include but is not limited to, supplier, biologic source, enzyme inducers, method of preparation, storage conditions, procedures followed during use, composition of the cofactor mix. Again, the author should present any information relevant to evaluation of the study design and interpretation of the results.

7.7 *Results*—Primary data sufficient to verify the results should be presented. If graphic presentations are included in a test report, they should be accompanied by numeric data. Transformed data should not be presented unless the method of transformation is clearly defined and sufficient primary data is presented to permit verification of the transformation. If the data are to be submitted to a regulatory agency, RTGs as well as VC and TFT plate counts should be presented for each treated culture as well as for solvent and positive control cultures.

7.8 *Data Evaluation*—The author should clearly state the data evaluation criteria and should apply appropriate statistical methodology where applicable.

7.9 *Study Acceptability*—For regulatory purposes, the criteria for acceptability of a study should be clearly stated. Any studies which are not acceptable by the author's criteria should be cited and the reason for rejection given.

7.10 *Good Laboratory Practices (GLP)*—The test report should clearly state if GLPs (Good Laboratory Practices of the FDA) or other quality assurance practices were followed. For regulatory purposes, this should also include a statement of quality assurance and audit procedures and any relevant findings.

7.11 *Conclusion*—The author's conclusion should be clearly and concisely stated.

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