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

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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.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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

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

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.

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**).

[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.

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 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.