

***In vitro* Mammalian Cell Gene Mutation Test
(L5178Y/TK^{+/-} Mouse Lymphoma Assay)**

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.0 SPONSOR

- 2.1 Name: **Perchlorate Study Group**
- 2.2 Address: Highway 50 and Aerojet Road
Building 20019/Department 0330
Rancho Cordova, CA 95813-6000
- 2.3 Study Monitor: Michael F. Girard
Perchlorate Study Group Representative
Telephone: (916) 355-2945
Telefax: (916) 355-6145
- 2.4 Scientific Advisor: Michael L. Dourson, Ph.D., DABT
Toxicology Excellence for Risk Assessment
4303 Hamilton Ave.
Cincinnati, OH 45223
Telephone: (513) 542-7475
Telefax: (513) 542-7487
- 2.5 Sponsor Project #:

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- 3.1 Test Article: **Ammonium perchlorate**
- 3.2 Controls: Negative: Test article solvent (or vehicle)
Positive: Methyl methanesulfonate (MMS)
7,12-dimethylbenz(a)anthracene (DMBA)
- 3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date:

5.2 Proposed Experimental Completion Date:

5.3 Proposed Report Date:

6.0 TEST SYSTEM

L5178Y/TK^{+/-} mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK^{+/-}, clone 3.7.2C, were received from Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, North Carolina. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination. This system has been demonstrated to be sensitive to the mutagenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutation assay will be performed by exposing duplicate cultures of L5178Y/TK^{+/-} cells to a minimum of eight concentrations of test article as well as positive and negative (solvent) controls. Exposures will be for 4 hours in the presence and absence of an S9 activation system. Following a two day expression period, with daily cell population adjustments, cultures demonstrating 0% to 90% growth inhibition will be cloned, in triplicate, in restrictive medium containing soft agar to select for the mutant phenotype. After a 10 to 14 day selection period, mutant colonies will be enumerated. The mutagenic potential of the test article will be measured by its ability to induce TK^{+/-} → TK^{-/-} mutations. For those test articles demonstrating a positive response, mutant colonies will be sized as an indication of mechanism of action.

7.1 Selection of Solvent

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to measure the maximum soluble concentration in a variety of solvents. Solvents compatible with this test system, in order of preference, include, but are not limited to, culture medium or distilled water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone CAS 67-64-1). The solvent of choice will be that solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

In the preliminary toxicity test, L5178Y/TK[±] cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium but not to exceed 5000 µg/ml. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. After a 4-hour treatment in the presence and absence of S9 activation, cells will be washed twice with F₀P (Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics) or F₁₀P (F₀P supplemented with 10% horse serum and 2mM L-glutamine) and cultured in suspension for two days post-treatment, with cell concentration adjustment on the first day.

Selection of dose levels for the mutation assay will be based on reduction of suspension growth after treatment in the preliminary toxicity test. Unless specified otherwise by the Sponsor, the high dose for the mutation assay will be that concentration exhibiting approximately 100% growth inhibition. The low dose will be selected to exhibit 0% growth inhibition. For freely soluble, non-toxic test articles, the highest concentration will be 5000 µg/ml. For relatively insoluble, non-toxic test articles, the highest concentration will be the lowest insoluble dose in treatment medium but not to exceed 5000 µg/ml. In all cases, precipitation will be evaluated at the beginning and at the end of the treatment period using the naked eye (ICH, 1996).

7.3 Route and Frequency of Administration

Cell cultures will be treated for 4 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The source of S9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid, 6 mg NADP, and 0.25 ml S9 homogenate per ml in F₀P. The S9 mix will be adjusted to pH 7.

7.5 Controls

7.5.1 Negative Control

The solvent (or vehicle) for the test article will be used as the negative control.

7.5.2 Positive Controls

Methyl methanesulfonate (MMS) will be used at two concentrations of 10 and 20 µg/ml as the positive control for the non-activated test system. For the S9-activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two concentrations of 2.5 and 4.0 µg/ml.

7.6 Preparation of Target Cells

Prior to use in the assay, L5178Y/TK^{+/-} cells will be cleansed to reduce the frequency of spontaneously occurring TK^{-/-} cells. Using the procedure described by Clive and Spector (1975), L5178Y cells will be cultured for 24 hours in the presence of thymidine, hypoxanthine, methotrexate and glycine to poison the TK^{-/-} cells.

L5178Y/TK^{+/-} cells will be prepared at 1 x 10⁶ cells/ml in 50% conditioned F₁₀P and 50% F₀P. If cultures are to be treated with more than 100 µl of test article dosing solution, the cell concentration may be adjusted.

7.7 Identification of the Test System

Using a permanent marking pen, the treatment tubes will be identified by the study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in conical tubes by combining 100 µl dosing solution of test or control article in solvent or solvent alone, 4 ml F₀P medium or S9 activation mixture with 6 x 10⁶ L5178Y/TK^{+/-} cells in a total volume of 10 ml. A minimum of eight concentrations of test article will be tested in duplicate. All pH adjustments will be performed prior to adding S9 or target cells to the treatment medium. Volumes of test article dosing solution in excess of 100 µl may be used if required to achieve the target final concentration in treatment medium. Treatment tubes will be gassed with 5±1% CO₂ in air, capped tightly, and incubated with mechanical mixing for 4 hours at 37±1°C. The preparation and addition of the test

article dosing solutions will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

7.9 Expression of the Mutant Phenotype

At the end of the exposure period, the cells will be washed twice with F₀P or F₁₀P and collected by centrifugation. The cells will be resuspended in 20 ml F₁₀P, gassed with 5±1% CO₂ in air and cultured in suspension at 37±1°C for two days following treatment. Cell population adjustments to 0.3 x 10⁶ cells/ml will be made at 24 and 48 hours.

7.10 Selection of the Mutant Phenotype

For selection of the trifluorothymidine (TFT)-resistant phenotype, cells from a minimum of five non-activated and five S9-activated test article concentrations demonstrating from 0% to 90% suspension growth inhibition will be plated into three replicate dishes at a density of 1 x 10⁶ cells/100mm plate in cloning medium containing 0.23% agar and 2-4 µg TFT/ml. For estimation of cloning efficiency at the time of selection, 200 cells/100mm plate will be plated in triplicate in cloning medium free of TFT (viable cell (VC) plate). Plates will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO₂ for 10-14 days.

The total number of colonies per plate will be determined for the VC plates and the total relative growth calculated. The total number of colonies per TFT plate will then be determined for those cultures with ≥10% total growth. Colonies are enumerated using an automatic counter; if the automatic counter cannot be used, the colonies will be counted manually. The diameters of the TFT colonies from the positive control and solvent control cultures will be determined over a range of approximately 0.2 to 1.1 mm. In the event the test article demonstrates a positive response, the diameters of the TFT colonies for at least one dose level of the test article (the highest positive concentration) will be determined over a range of approximately 0.2 to 1.1 mm.

7.11 Independent Repeat Assay

Verification of a clear positive response will not be required (OECD Guideline 476, ICH 1997). For equivocal and negative results, the Sponsor will be consulted regarding the need for an independent repeat assay.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The spontaneous mutant frequency of the solvent (or vehicle) control cultures must be within 20 to 100 TFT-resistant mutants per 10⁶ surviving cells. The cloning efficiency of the solvent (or vehicle) control group must be greater than 50%.

8.2 Positive Controls

At least one concentration of each positive control must exhibit mutant frequencies of ≥ 100 mutants per 10^6 clonable cells over the background level. The colony size distribution for the MMS positive control must show an increase in both small and large colonies (Moore *et al.*, 1985; Aaron *et al.*, 1994).

8.3 Test Article-Treated Cultures

A minimum of four analyzable concentrations with mutant frequency data will be required.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT-plates by the mean number of colonies on the VC-plates and multiplying by the dilution factor (2×10^{-4}), and is expressed as TFT-resistant mutants per 10^6 surviving cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations (i.e., less than 10% total growth) are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data (Clive *et al.*, 1995):

- The result will be considered to induce a positive response if a concentration-related increase in mutant frequency is observed and one or more dose levels with 10% or greater total growth exhibit mutant frequencies of ≥ 100 mutants per 10^6 clonable cells over the background level.
- A result will be considered equivocal if the mutant frequency in treated cultures is between 55 and 99 mutants per 10^6 clonable cells over the background level.
- Test articles producing fewer than 55 mutants per 10^6 clonable cells over the background level will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

Results presented will include, but not be limited to:

- test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- solvent/vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO₂ concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant colonies and the number of colonies in each plate
- dose-response relationship, if applicable
- distribution of the mutant colony diameter for the solvent and positive controls and, when the test article induces a positive response, for at least one dose level of the test article (the highest positive concentration)
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of BioReliance, Rockville, MD in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), July 1997, and with the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995, Federal Register 61:18198-18202, April 24, 1996.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? _____

If so, to which agency or agencies? _____

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Aaron, C.S., Bolcsfoldi, G., Glatt, H.-R., Moore, M., Nishi, Y., Stankowski, L., Theiss, J. and Thompson, E. (1994) Mammalian cell gene mutation assays working group report. *Mutation Research* 312:235-239.

Clive, D., Bolcsfoldi, G., Clements, J., Cole, J., Homna, M., Majeska, J., Moore, M., Muller, L., Myhr, B., Oberly, T., Oudelhkim, M., Rudd, C., Shimada, H., Sofuni, T., Thybaud, V. and Wilcox, P. (1995) Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. *Environ. Molec. Mutagen.* 25:165-168.

Clive, D. and Spector, J.F.S. (1975) Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Research* 31:17-29.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. *Federal Register* 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. *Federal Register* 62:16026-16030, November 21, 1997.

Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. and Turner, N.T. In situ analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK^{+/-} mouse lymphoma cells. (1985) *Mutation Research* 151:147-159.

OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), July 1997.

14.0 APPROVAL

Michael F. Girard
Sponsor Study Monitor

Date

Michael L. Dourson, Ph.D., DABT
Sponsor Scientific Advisor

Date

Richard H.C. San, Ph.D.
BioReliance Study Director

Date

If submission to Japanese Regulatory Agency is indicated in section 12.0,
BioReliance management will sign.

David Jacobson-Kram, Ph.D., DABT
BioReliance Study Management

Date