TRADE SECRET

Study Title

H-28548: *In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK+/- Mouse Lymphoma Assay)

Testing Guidelines

ICH S2A document April 24, 1996
ICH S2B document November 21, 1997
OECD Guideline for the Testing of Chemicals, Guideline 476 (1998)
US EPA Health Effects Test Guidelines, OPPTS 870.5300 (1998)
EC Commission Directive 2000/32/EC, Annex 4E No. L136

Author
Jane J. Clarke, M.S.

Study Completion Date
25 June 2008

Testing Facility
BioReliance
9630 Medical Center Drive
Rockville, MD 20850, USA
for
E. I. du Pont de Nemours and Company
DuPont Haskell Global Centers for Health and Environmental Sciences
P.O. Box 50
1090 Elkton Road
Newark, DE 19714-0050, USA

BioReliance Study Number
AC15UX.704.BTL

Work Request Number
17751

Service Code
1537

STATEMENT OF COMPLIANCE

Study No. AC15UX.704.BTL, was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the OECD (C(97) 186/FINAL) Principles of Good Laboratory Practice, and the US EPA GLP Standards 40 CFR 792 in all material aspects with the following exception:

> Analyses to determine the uniformity or concentration of the test article and control mixtures and their stability were not performed by the testing facility or the Sponsor.

Applicant/Sponsor: E. I. du Pont de Nemours and Company
Wilmington, DE 19898, USA

BioReliance Study Director: _____ 25 June 2008
Jane J. Clarke, M.S. Date

BioReliance Study Management: _____ 25 June 2008
Date

Applicant/Sponsor: _____ _____
DuPont Representative Date

Quality Assurance Statement

Study Title: H-28548: *In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK+/- Mouse Lymphoma Assay)

Study Number: AC15UX.704.BTL

Study Director: Jane J. Clarke, M.S.

Quality Assurance performed the inspections listed below for this study. Verification of the study protocol was also performed and documented by QA. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the OECD Principles of Good Laboratory Practice, the U.S. EPA Good Laboratory Standards 40 CFR 792, and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On: 09-Jun-08 - 09-Jun-08 To Study Dir 09-Jun-08 To Mgmt 09-Jun-08
Phase: Recording data into MLS system

Inspect On: 12-Jun-08 - 13-Jun-08 To Study Dir 13-Jun-08 To Mgmt 13-Jun-08
Phase: Draft Report and Data Audit

Inspect On: 25-Jun-08 - 25-Jun-08 To Study Dir 25-Jun-08 To Mgmt 25-Jun-08
Phase: Draft to Final Report

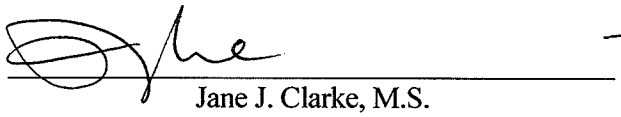
This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Marcie Winters 25 Jun 2008

Marcie Winters, BS DATE
QUALITY ASSURANCE

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director:  25 June 2008

Jane J. Clarke, M.S. Date
BioReliance

Approved by
Study Sponsor:

E. Maria Donner, Ph.D.
Senior Research Toxicologist and Manager
E.I. du Pont de Nemours and Company
DuPont Haskell Global Centers for Health and
Environmental Sciences
P.O. Box 50
1090 Elkton Road
Newark, DE 19714-0050, USA

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STUDY INFORMATION

Substance Tested:
- HFPO Dimer Acid Ammonium Salt
- 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, ammonium salt
- 62037-80-3 (CAS Number)
- H-28548

Haskell Number: 28548

Composition:

84%	HFPO Dimer Acid Ammonium Salt
12.7%	Water
150 ppm	Perfluorooctanoic acid

Purity: 86.6%

Physical Characteristics: Clear and colorless liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: 15 May 2008/ (see report cover page)

Experimental Start/Termination: 19 May 2008 / 09 June 2008

SUMMARY

The test article, H-28548, was tested in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor-induced rat liver S9. The preliminary toxicity assay was used to establish the concentration range for the mutagenesis assay. The mutagenesis assay was used to evaluate the mutagenic potential of the test article. The dosing formulations were adjusted to compensate for the purity of the test article using a correction factor of 1.15.

Sterile distilled water was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in water at approximately 50 mg/mL, the maximum concentration tested.

In the preliminary toxicity assay, the maximum concentration of H-28548 in treatment medium was 3500 µg/mL (10 mM). No visible precipitate was present at any concentration in treatment medium. Selection of concentrations for the mutation assay was based on reduction of suspension growth relative to the solvent control. Substantial toxicity, i.e., suspension growth of ≤ 50% of the solvent control, was observed at 3500 µg/mL without activation with a 4-hour exposure, ≥ 1500 µg/mL with S9 activation, and ≥ 1500 µg/mL without activation with a 24-hour exposure.

Based on the results of the preliminary toxicity assay, the concentrations tested in the initial mutagenesis assay ranged from 500 to 3500 µg/mL for the non-activated cultures and 150 to 2000 µg/mL for the S9-activated cultures with a 4-hour exposure. Visible precipitate was not present at any concentration in treatment medium. The concentrations chosen for cloning were 500, 750, 1000, 1500, and 2000 µg/mL without activation and 150, 250, 500, 600, and 750 µg/mL with S9 activation. No cloned cultures exhibited mutant frequencies ≥ 90 mutants per 10^6 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

Based on the results of the preliminary toxicity assay, the concentrations tested in the extended treatment assay ranged from 150 to 2000 µg/mL for non-activated cultures with a 24-hour exposure. Visible precipitate was not present at any concentration in treatment medium. The concentrations chosen for cloning were 250, 500, 600, 750, and 1000 µg/mL. No cloned cultures exhibited mutant frequencies ≥ 90 mutants per 10^6 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

The trifluorothymidine-resistant colonies for the positive and solvent control cultures from both assays were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the positive controls yielded the expected increase in small colonies (verifying the adequacy of the methods used to detect small colony mutants) and large colonies.

All criteria for a valid test were met. Under the conditions of this study, test article H-28548 was concluded to be negative in the presence and absence of S9 metabolic activation in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay. The assay was negative.

PURPOSE

The purpose of this study was to evaluate the genotoxic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells and the sizing of the resulting colonies according to the protocol in Appendix B.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test article, H-28548, was received by BioReliance on 15 May 2008 and was assigned the code number AC15UX. The test article was described by the Sponsor as a liquid, which should be stored at ambient temperature in the dark. A purity of 86.6% was provided in the protocol. The purity stated on the Certificate of Analysis, provided after the conclusion of the study, was 84%. The Sponsor does not consider this small discrepancy in purity to have any effect on the integrity or outcome of the study. (This is particularly true since the top concentration was driven by precipitation.) A reanalysis date of 13 June 2009 was provided. Upon receipt, the test article was described as a clear colorless liquid and was stored at room temperature protected from light.

The Sponsor has determined the identity, strength, purity, and composition or other characteristics to define the test article. A copy of the Certificate of Analysis is included in Appendix C. Based on the reanalysis date in the Certificate of Analysis, the test article is considered stable for the purpose of this study through 13 June 2009.

The vehicle (solvent) used to deliver H-28548 to the test system was sterile distilled water (CAS 7732-18-5), lot 1391332, expiration January 2009, obtained from Gibco. The dosing solutions were adjusted to compensate for the purity of the test article using the correction of 1.15 provided by the Sponsor. The test article dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light.

Methyl methanesulfonate (MMS), CAS 66-27-3, lot #05713JD, expiration date 06 November 2008, supplied by Aldrich Chemical Company was diluted in sterile water, CAS 7732-18-5, lot # 1391332, expiration date January 2009 and used as the positive control for the non-activated test system, and to determine that the assay is capable of detecting small colonies, at stock concentrations of 1500 and 2000 μg/mL with a 4-hour exposure or 500 and 750 μg/mL with a 24-hour exposure. 7,12-Dimethyl-benz(a)anthracene (7,12-DMBA), CAS 57-97-6, lot # 055K1360, expiration date 20 September 2010, supplied by Sigma Chemical Company was diluted in DMSO (CAS 67-68-5), lot 47232745, expiration 14 December 2010, obtained from EMD Chemicals and used at stock concentrations of 100 and 125 μg/mL as the positive control for the S9-activated test system.

The negative and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control articles and their respective mixtures was demonstrated by acceptable results that met the criteria for a valid test. Historical control data are presented in Appendix A.

MATERIALS AND METHODS

Test System

L5178Y cells, clone 3.7.2C, were obtained from Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, NC on 14 August 1995. Each lot of cryopreserved cells was tested using the agar culture and Hoechst staining procedures and found to be free of mycoplasma contamination. Prior to use in the assay, L5178Y cells were cleansed of spontaneous TK$^{-/-}$ cells by culturing in a restrictive medium (Clive and Spector, 1975).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 lot 2178, was purchased by BioReliance from Moltox (Boone, NC) and stored at ≤ -60°C until used. Each lot of S9 was assayed for sterility and its ability to metabolize at least two pro-mutagens to forms mutagenic to *Salmonella typhimurium* TA100. The Record of Analysis is on file with the testing facility.

Immediately prior to use, the S9 was mixed with the cofactors and Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F_0P) to contain 25 µL S9, 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), 11.25 mg DL-isocitric acid, and 975 µL F_0P per mL S9-activation mixture and kept on ice until used. The cofactor/F_0P mixture was adjusted to pH 7.0 and filter-sterilized prior to the addition of S9.

Solubility Test

A solubility test was conducted to select the vehicle. The test article was tested to determine the vehicle which permitted preparation of the highest soluble or workable stock concentration, up to 50 mg/mL in water.

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the optimal concentrations for the mutagenesis assay. L5178Y cells were exposed to the solvent alone and nine concentrations of test article ranging from 0.5 to 3500 µg/mL in both the absence and presence of S9 activation with a 4-hour exposure and without activation with a 24-hour exposure. The osmolality of the solvent control and the highest soluble concentration in treatment medium were determined. The pH of the cultures was adjusted to neutral prior to the addition of cells and S9 mix.

For the 4-hour exposure, cell population density was determined 24 and 48 hours after the exposure to the test article; the cultures were adjusted to $3x10^5$ cells/mL after 24 hours only. For the 24-hour exposure, cell population density was determined 24, 48, and 72 hours after the exposure to the test article. The cell population was adjusted to 3×10^5 cells/mL immediately after test article removal

and 24 hours after test article removal. Cultures with less than $3x10^5$ cells/mL were not adjusted. Toxicity was measured as suspension growth of the treated cultures relative to the growth of the solvent control cultures after 48 hours.

Mutagenesis Assays

The mutagenesis assay (with and without S9 activation with a 4-hour exposure) and extended treatment assay (without S9 activation with a 24-hour exposure) were used to evaluate the mutagenic potential of the test article. L5178Y mouse lymphoma cells were exposed to the solvent alone and eight concentrations of test article in duplicate in both the absence and presence of S9. Positive controls, with and without S9 activation, were tested concurrently. The pH of the cultures was adjusted to neutral prior to the addition of cells and S9 mix.

Treatment of the Target Cells

The mutagenesis assay was performed according to a protocol described by Clive and Spector (1975). Treatment was carried out in conical tubes by combining 6 x 10^6 L5178Y/TK$^{+/-}$ cells, F_0P medium or S9 activation mixture, and 1.0 mL dosing solution of test article in solvent or solvent alone in a total volume of 10 mL. The positive controls were treated with 100 µL MMS (at final concentrations in treatment medium of 15 and 20 µg/mL with a 4-hour exposure or 5.0 and 7.5 µg/ml with a 24-hour exposure) or 7,12-DMBA (at final concentrations in treatment medium of 1.0 and 1.25 µg/mL). Treatment tubes were gassed with 5±1% CO_2 in air, capped tightly, and incubated with mechanical mixing for 4 or 24 hours at 37±1°C. The preparation and addition of the test article dosing solutions were carried out under amber lighting and the cells were incubated in the dark during the exposure period. After the treatment period, the cells were washed twice with F_0P or F_0P supplemented with 10% horse serum, 2 mM L-glutamine, 100 U penicillin/mL and 100 µg streptomycin/mL ($F_{10}P$). After the second wash, the cells were resuspended in $F_{10}P$, gassed with 5±1% CO_2 in air and placed on the roller drum apparatus at 37±1°C.

Expression of the Mutant Phenotype

For expression of the mutant phenotype, the cultures were counted using an electronic cell counter and adjusted to $3x10^5$ cells/mL at approximately 24 and 48 hours after treatment in 20 and 10 mL total volume, respectively. For the 24-hour exposure, cultures were adjusted to $3x10^5$ cells/mL in 20 mL immediately after test article removal, then at 48 and 72 hours after treatment in 20 and 10 mL total volume, respectively. Cultures with less than $3x10^5$ cells/mL were not adjusted.

For expression of the TK$^{-/-}$ cells, cells were placed in cloning medium (C.M.) containing 0.22% dissolved Noble agar in F_0P plus 20% horse serum. Two flasks per culture to be cloned were labeled with the test article concentration, activation condition, and either TFT (trifluorothymidine, the selective agent) or VC (viable count). Each flask was prewarmed to 37±1ºC, filled with 100 mL C.M., and placed in an incubator shaker at 37±1°C until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were then diluted in C.M. to concentrations of $3x10^6$ cells/100 mL C.M. for the TFT flask and 600 cells/100 mL C.M. for the VC flask. After the dilution, 1.0 mL of stock solution of TFT was added to the TFT flask (final

concentration of 3 μg/mL) and both this flask and the VC flask were placed on the shaker at 125 rpm and 37±1°C. After 15 minutes, the flasks were removed and the cell suspension was divided equally into each of three appropriately labeled Petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 30 minutes. The plates were then incubated at 37±1°C in a humidified 5±1% CO_2 atmosphere for 10-14 days.

Scoring Procedures

After the incubation period, the VC plates were counted for the total number of colonies per plate and the total relative growth determined. The TFT-resistant colonies were then counted for each culture with ≥ 20% total relative growth (including at least one concentration with ≥ 10% but ≤ 20% total growth). The diameters of the TFT-resistant colonies for the positive and solvent controls and, in the case of a positive response, the test article-treated cultures were determined over a range of approximately 0.2 to 1.1 mm. The rationale for this procedure is as follows: Mutant L5178Y TK$^{-/-}$ colonies exhibit a characteristic frequency distribution of colony sizes. The precise distribution of large and small TFT-resistant mutant colonies appears to be the characteristic mutagenic "finger-print" of carcinogens in the L5178Y TK$^{+/-}$ system (Clive *et al*., 1979; DeMarini *et al*., 1989). Clive *et al*. (1979) and Hozier *et al*. (1981) have presented evidence to substantiate the hypothesis that the small colony variants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse. They suggested that large colony mutants received very localized damage, possibly in the form of a point mutation or small deletion within the TK locus, while small colony mutants received damage to collateral loci concordant with the loss of TK activity.

Criteria for a Valid Test

The following criteria must be met for the mutagenesis assay to be considered valid:

Negative Controls

The average spontaneous mutant frequency of the solvent (or vehicle) control cultures must be within 35 to 140 TFT-resistant mutants per 10^6 surviving cells. Low spontaneous mutant frequencies, i.e., 20 to 34 mutants per 10^6 surviving cells, are considered acceptable if small colony recovery is demonstrated (Mitchell et al., 1997). The average cloning efficiency of the solvent (or vehicle) controls must be between 65% and 120% and the total suspension growth between 8-32 for the 4-hour exposure and 20-180 for the 24-hour exposure (Moore, et al., 2002 and 2006).

Positive Controls

The mutant frequency for at least one dose of the positive controls must meet the criteria for a positive response and induce an increase in small colony mutants according to the following criteria: Induced Mutant Frequency (IMF) positive control ≥ 300 x 10^{-6} mutants with 40% small colonies **or** small colony IMF for positive control ≥ 150 x 10^{-6} (Moore, *et al.*, 2002; 2006).

Test Substance-Treated Cultures:

Cultures treated with a minimum of four concentrations of test article must be attained and their mutant frequencies reported. The highest test article concentration must produce 80% to 90% toxicity (ICH, 1996) unless limited by solubility or the maximum required concentration as described in section 7.2 of the protocol. In the case of a test article with a steep toxicity curve (no concentrations with 10-20% survival), the results may be considered acceptable if a concentration spacing of $\leq$ 2-fold is used and the highest concentration tested showed <20% survival or total kill (Sofuni *et al.*, 1997). For example, the test is considered acceptable if the highest concentration cloned for mutant selection exhibits >20% survival and the next highest concentration, which is $\leq$ 2 times the cloned concentration, is too toxic to clone.

Evaluation of Results

The cytotoxic effects of each treatment condition were 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 (number of mutants per 10^6 surviving cells) for each treatment condition was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding VC plates and multiplying by the dilution factor ($2x10^{-4}$) then multiplying by 10^6. For simplicity, this is described as: (Average # TFT colonies / average # VC colonies) x 200 in the tables.

In evaluation of the data, increases in mutant frequencies that occurred only at highly toxic concentrations (i.e., less than 10% total growth) were not considered biologically relevant. All conclusions were based on scientific judgment; however, the following criteria are presented as a guide to interpretation of the data (Moore et al., 2006):

- A result was considered positive if a concentration-related increase in mutant frequency was observed in the treated cultures and one or more treatment conditions with 10% or greater total growth exhibited mutant frequencies of ≥90 mutants per 10^6 clonable cells over the background level (based on the average mutant frequency of duplicate cultures). If the average solvent control mutant frequency was >90 mutants per 10^6 clonable cells, a doubling of mutant frequency over the background will also be required (Mitchell *et al.*, 1997).

- A result was considered negative if the treated cultures exhibited mutant frequencies of less than 90 mutants per 10^6 clonable cells over the background level (based on the average mutant frequency of duplicate cultures) and there was no concentration-related increase in mutant frequency.

- There are some situations where a chemical would be considered negative when there was no culture showing between 10-20% survival: 1) There was no evidence of mutagenicity (e.g. no dose response or increase in mutant frequencies between 45 and 89 mutants per 10^6 above control) in a series of data points within 100% to 20% survival *and* there was at least one

negative data point between 20% and 25% survival. 2) There was no evidence of mutagenicity (e.g. no dose response or increase in mutant frequencies between 45 and 89 mutants per 10^6 above control) in a series of data points between 100% to 25% survival *and* there was also a negative data point between 10% and 1% survival (Office of Food Additive Safety, 2001). In this case it would be acceptable to count the TFT colonies of cultures exhibiting <10% total growth.

Records and Archives

All raw data, protocol, and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years. Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

Deviations

No deviations from the protocol occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Sterile distilled water was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in water at approximately 50 mg/mL, the maximum concentration tested.

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in Table 1. The maximum concentration tested in the preliminary toxicity assay was 3500 µg/mL (10 mM). Visible precipitate was not present at any concentration in treatment medium. The osmolality of the solvent control was 245 mmol/kg and the osmolality of the highest soluble concentration, 3500 µg/mL, was 260 mmol/kg. The pH of the cultures treated with 1500 and 3500 µg/mL was adjusted to neutral with 1N hydrochloric acid. Suspension growth relative to the solvent controls was 0% at 3500 µg/mL without activation with a 4-hour exposure and at ≥ 1500 µg/mL with S9 activation with a 4-hour exposure and without activation with a 24-hour exposure. Based on the results of the toxicity test, the concentrations tested in the mutagenesis assay ranged from 500 to 3500 µg/mL for the non-activated cultures with a 4-hour exposure and 150 and 2000 µg/mL for the S9-activated cultures with a 4-hour exposure and non-activated cultures with a 24-hour exposure.

Mutagenesis Assays

The results of the initial mutagenesis assay are presented in Tables 2 and 3. Colony size distributions for the positive and solvent control cultures are presented in Figures 1 and 2. The pH of the cultures treated with 1500, 2000, 2500, 3000, and 3500 µg/mL was adjusted to neutral with 1N hydrochloric acid. No visible precipitate was present at any concentration in treatment medium. In the non-activated system, cultures treated with concentrations of 500, 750, 1000, 1500, and 2000 µg/mL were cloned and produced a range in suspension growth from 13% to 92%. In the S9-activated system, cultures treated with concentrations of 150, 250, 500, 600, and 750 µg/mL were cloned and produced a range in suspension growth from 20% to 102%.

No cloned cultures exhibited mutant frequencies ≥ 90 mutants per 10^6 clonable cells over that of the solvent control. No concentration-related increase in mutant frequency was observed. The total growth ranged from 12% to 95% for the non-activated cultures at concentrations from 500 to 2000 µg/mL and 20% to 100% for the S9-activated cultures at concentrations from 150 to 750 µg/mL.

The results of the initial assay were negative in the absence and presence of S9 activation. Because no unique metabolic requirements were known about the test article, only an extended treatment assay in the absence of S9 for a 24-hour exposure period was performed.

The results of the extended treatment assay are presented in Table 4. Colony size distributions for the positive and solvent control cultures are presented in Figure 3. No visible precipitate was present at any concentration in treatment medium. Cultures treated with concentrations of 250, 500, 600, 750, and 1000 µg/mL were cloned and produced a range in suspension growth from 29% to 99%.

No cloned cultures exhibited mutant frequencies ≥ 90 mutants per 10^6 clonable cells over that of the solvent control. No concentration-related increase in mutant frequency was observed. The total growth ranged from 31% to 117% for non-activated cultures with a 24-hour exposure at concentrations from 250 to 1000 µg/mL.

The trifluorothymidine-resistant colonies for the positive and solvent control cultures from the mutation assay were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the MMS and DMBA positive controls yielded the expected increase in small colonies (verifying the adequacy of the methods used to detect small colony mutants) and large colonies.

CONCLUSION

All criteria for a valid study were met as described in the protocol. Under the conditions of this study, test article H-28548 was concluded to be negative in the absence and presence of S9 metabolic activation in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay. The assay was negative.

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TABLE 1
PRELIMINARY TOXICITY ASSAY, 4-Hour Exposure

DOSE LEVEL (µg/mL)	PRECIP	CELL CONCENTRATION (cells/mL x 10^6)			SUSPENSION GROWTH	
		DAY 0	DAY 1	DAY 2	TOTAL	% OF CONTROL
4HR NON-ACTIVATED CULTURES						
SOLVENT 1		Not applicable for 4-hour exposure	1.252	1.524	21.2	**100**
SOLVENT 2			1.118	1.451	18.0	
0.5			1.239	1.524	21.0	**107**
1.5			1.250	1.447	20.1	**103**
5			1.238	1.486	20.4	**104**
15			1.282	1.440	20.5	**105**
50			1.320	1.214	17.8	**91**
150			1.233	1.371	18.8	**96**
500			1.231	1.410	19.3	**99**
1500			0.789	1.150	10.1	**52**
3500			0.149	0.132	0.0	**0**
4HR S9-ACTIVATED CULTURES **(Induced Rat Liver S9)**						
SOLVENT 1		Not applicable for 4-hour exposure	1.105	1.609	19.7	**100**
SOLVENT 2			1.096	1.735	21.1	
0.5			1.093	1.588	19.3	**94**
1.5			1.088	1.595	19.3	**94**
5			1.075	1.603	19.1	**94**
15			1.087	1.561	18.9	**92**
50			1.126	1.506	18.8	**92**
150			1.007	1.578	17.7	**86**
500			0.854	1.511	14.3	**70**
1500			0.014	0.021	0.0	**0**
3500			0.031	0.032	0.0	**0**

Solvent = water 1 and 2 are duplicate cultures

Cultures containing <0.3x106 cells/mL on day 0, 1, and 2 are considered to have 0% total suspension growth.

$$\text{Total suspension growth (4-hr)} = \frac{\text{Day 0 cell conc.}}{0.3\text{x}10^6 \text{ cells/mL}} \times \frac{\text{Day 1 cell conc.}}{\text{Day 0 adjusted cell conc.}}$$

$$\text{\% of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

TABLE 1 (cont.)
PRELIMINARY TOXICITY ASSAY, 24-Hour exposure

DOSE LEVEL (µg/mL)	PRECIP	CELL CONCENTRATION (cells/mL x 10^6)			SUSPENSION GROWTH	
		DAY 0	DAY 1	DAY 2	TOTAL	% OF CONTROL
24HR NON-ACTIVATED CULTURES						
SOLVENT 1		0.799	0.952	1.396	39.3	**100**
SOLVENT 2		0.759	0.982	1.363	37.7	
0.5		0.847	0.943	1.436	42.5	**110**
1.5		0.806	0.879	1.304	34.2	**89**
5		0.821	1.058	1.360	43.7	**114**
15		0.817	1.016	1.420	43.7	**114**
50		0.781	1.010	1.367	39.9	**104**
150		0.776	0.941	1.459	39.4	**102**
500		0.795	0.886	1.380	36.0	**94**
1500		0.076	0.113	0.293	0.0	**0**
3500		0.053	0.053	0.041	0.0	**0**

Solvent = water

1 and 2 are duplicate cultures

Cultures containing <0.3x106 cells/mL on day 0, 1, and 2 are considered to have 0% total suspension growth.

$$\text{Total suspension growth (24-hr)} = \frac{\text{Day 0 cell conc.}}{\text{0.3x106 cells/mL}} \times \frac{\text{Day 1 cell conc.}}{\text{Day 0 adjusted cell conc.}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$\text{\% of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

TABLE 2
DATA SUMMARY FOR THE INITIAL ASSAY (4-hour exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1		22.0	100	25	27	30	**27**	179	141	114	**145**	**38**	N/A	100
SOLVENT 2		22.7		34	38	36	**36**	170	145	194	**170**	**42**		
500 A		17.1	76	41	41	44	**42**	145	132	140	**139**	60	**20**	**68**
500 B		20.6	92	36	36	44	**39**	138	151	197	**162**	48	**8**	**95**
750 A		16.1	72	37	43	29	**36**	177	194	174	**182**	40	**0**	**83**
750 B		18.7	83	22	22	29	**24**	175	147	199	**174**	28	**-12**	**92**
1000 A		16.6	74	29	23	25	**26**	174	158	160	**164**	31	**-9**	**77**
1000 B		16.8	75	33	38	47	**39**	165	158	203	**175**	45	**5**	**84**
1500 A		6.9	31	43	33	28	**35**	164	123	152	**146**	47	**7**	**29**
1500 B		8.4	37	22	30	34	**29**	155	183	210	**183**	31	**-9**	**43**
2000 A		2.7	13	25	28	19	**24**	156	144	131	**144**	33	**-7**	**12**
2000 B		4.4	20	19	24	25	**23**	165	178	160	**168**	27	**-13**	**21**
2500 A		1.4	8	+				+						
2500 B		0.9	7	+				+						
POSITIVE CONTROL:		**Methyl methanesulfonate (MMS)**								**(µg/mL)**				
20		10.0	45	88	83	100	**90**	39	41	29	**36**	497	**457**	**10**
15		12.8	57	113	99	151	**121**	51	60	74	**62**	392	**352**	**22**

MEAN SOLVENT TOTAL SUSPENSION GROWTH: 22.3
MEAN SOLVENT CLONING EFFICIENCY: 79%
MEAN SOLVENT MUTANT FREQUENCY: 40 (PER 10^6 CELLS)

Solvent = water A and B or 1 and 2 are duplicate cultures + - Too toxic to clone

$$\text{Mutant frequency per } 10^6 \text{ surviving cells} = \frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$$

$$\text{Induced mutant frequency per } 10^6 \text{ surviving cells} = \text{mutant frequency} - \text{average mutant frequency of solvent controls}$$

$$\text{Total suspension growth} = \frac{\text{Day 0 cell conc.}}{0.3\text{x}10^6 \text{ cells/mL}} \times \frac{\text{Day 1 cell conc.}}{\text{Day 0 adjusted cell conc.}}$$

$$\text{\% of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$\text{\% control cloning growth (not shown)} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$\text{\% total growth} = \frac{(\text{\% suspension growth})(\text{\% cloning growth})}{100}$$

TABLE 3
DATA SUMMARY FOR THE INITIAL ASSAY (4-hour exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1		19.0	100	38	53	70	**54**	152	163	185	**167**	64	N/A	100
SOLVENT 2		18.3		57	47	50	**51**	159	151	177	**162**	63		
150 A		17.5	94	27	34	51	**37**	147	124	151	**141**	53	**-11**	**80**
150 B		19.1	102	43	44	39	**42**	158	171	152	**160**	52	**-11**	**100**
250 A		16.3	87	47	66	53	**55**	178	147	126	**150**	74	**10**	**80**
250 B		15.9	85	62	51	51	**55**	170	158	133	**154**	71	**7**	**80**
500 A		13.0	70	46	39	47	**44**	178	142	147	**156**	57	**-7**	**66**
500 B		10.9	58	28	28	51	**36**	133	158	177	**156**	46	**-18**	**55**
600 A		10.4	56	*	43	39	**41**	164	177	164	**168**	49	**-15**	**57**
600 B		10.6	57	29	25	25	**26**	141	130	136	**136**	39	**-25**	**47**
750 A		4.6	25	42	43	29	**38**	163	144	149	**152**	50	**-14**	**23**
750 B		3.1	20	43	32	56	**44**	185	121	174	**160**	55	**-9**	**20**
1000 A		0.1	0	+				+						
1000 B		0.1	0	+				+						
POSITIVE CONTROL:		**7,12-dimethylbenz(a)anthracene (DMBA)**									**(µg/mL)**			
1.25		2.4	15	213	221	213	**216**	113	113	113	**113**	382	**318**	**10**
1		4.9	26	185	234	235	**218**	112	127	156	**132**	331	**267**	**21**

MEAN SOLVENT TOTAL SUSPENSION GROWTH: 18.6
MEAN SOLVENT CLONING EFFICIENCY: 82%
MEAN SOLVENT MUTANT FREQUENCY: 64 *(PER 10^6 CELLS)*

Solvent = water
* - plate lost to contamination

A and B or 1 and 2 are duplicate cultures
+ - Too toxic to clone

$$\text{Mutant frequency per } 10^6 \text{ surviving cells} = \frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$$

$$\text{Induced mutant frequency per } 10^6 \text{ surviving cells} = \text{mutant frequency} - \text{average mutant frequency of solvent controls}$$

$$\text{Total suspension growth} = \frac{\text{Day 0 cell conc.}}{0.3\times10^6 \text{ cells/mL}} \times \frac{\text{Day 1 cell conc.}}{\text{Day 0 adjusted cell conc.}}$$

$$\text{\% of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$\text{\% control cloning growth (not shown)} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$\text{\% total growth} = \frac{(\text{\% suspension growth})(\text{\% cloning growth})}{100}$$

TABLE 4
DATA SUMMARY FOR THE EXTENDED TREATMENT ASSAY (24-hour exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1		42.6	100	17	25	19	**20**	154	128	128	**137**	30	N/A	100
SOLVENT 2		43.5		14	18	29	**20**	122	132	165	**140**	29		
250 A		41.4	96	15	25	18	**19**	147	164	166	**159**	24	**-5**	**111**
250 B		42.5	99	20	13	19	**17**	164	155	173	**164**	21	**-8**	**117**
500 A		31.8	74	19	24	23	**22**	144	198	221	**188**	23	**-6**	**100**
500 B		33.7	78	17	29	27	**24**	184	161	217	**187**	26	**-3**	**106**
600 A		31.8	74	19	28	25	**24**	154	164	160	**159**	30	**1**	**85**
600 B		32.3	75	18	30	29	**26**	118	140	154	**137**	37	**8**	**74**
750 A		28.0	65	29	20	19	**23**	137	178	122	**146**	31	**2**	**68**
750 B		27.0	63	18	18	20	**19**	164	151	192	**169**	22	**-7**	**77**
1000 A		12.7	29	18	20	28	**22**	127	166	138	**144**	31	**1**	**31**
1000 B		15.1	35	29	30	33	**31**	149	170	197	**172**	36	**6**	**44**
1500 A		0.0	0	+				+						
1500 B		0.0	0	+				+						
POSITIVE CONTROL:		**Methyl methanesulfonate (MMS)**								**(µg/mL)**				
7.5		16.8	39	182	171	180	**178**	46	48	75	**56**	631	**601**	**16**
5		25.2	58	144	193	197	**178**	95	99	121	**105**	339	**310**	**44**
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 43.1														
MEAN SOLVENT CLONING EFFICIENCY: 69%														
MEAN SOLVENT MUTANT FREQUENCY: 29 (PER 10^6 CELLS)														

Solvent = water A and B or 1 and 2 are duplicate cultures + - Too toxic to clone

$$\text{Mutant frequency per } 10^6 \text{ surviving cells} = \frac{\text{Average \# TFT colonies}}{\text{average \# VC colonies}} \times 200$$

$$\text{Induced mutant frequency per } 10^6 \text{ surviving cells} = \text{mutant frequency} - \text{average mutant frequency of solvent controls}$$

$$\text{Total suspension growth} = \frac{\text{Day 0 cell conc.}}{0.3\times10^6 \text{ cells/mL}} \times \frac{\text{Day 1 cell conc.}}{\text{Day 0 adjusted cell conc.}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

$$\text{\% of control suspension growth} = \frac{\text{total treatment suspension growth}}{\text{average solvent control total suspension growth}} \times 100$$

$$\text{\% control cloning growth (not shown)} = \frac{\text{average VC of treated culture}}{\text{average VC of solvent control}} \times 100$$

$$\text{\% total growth} = \frac{(\text{\% suspension growth})(\text{\% cloning growth})}{100}$$

Figure 1
Initial Assay without Activation, 4-Hour Exposure
Colony Size Distribution in the Absence of Metabolic Activation
(Positive Control Compared with Solvent Control)

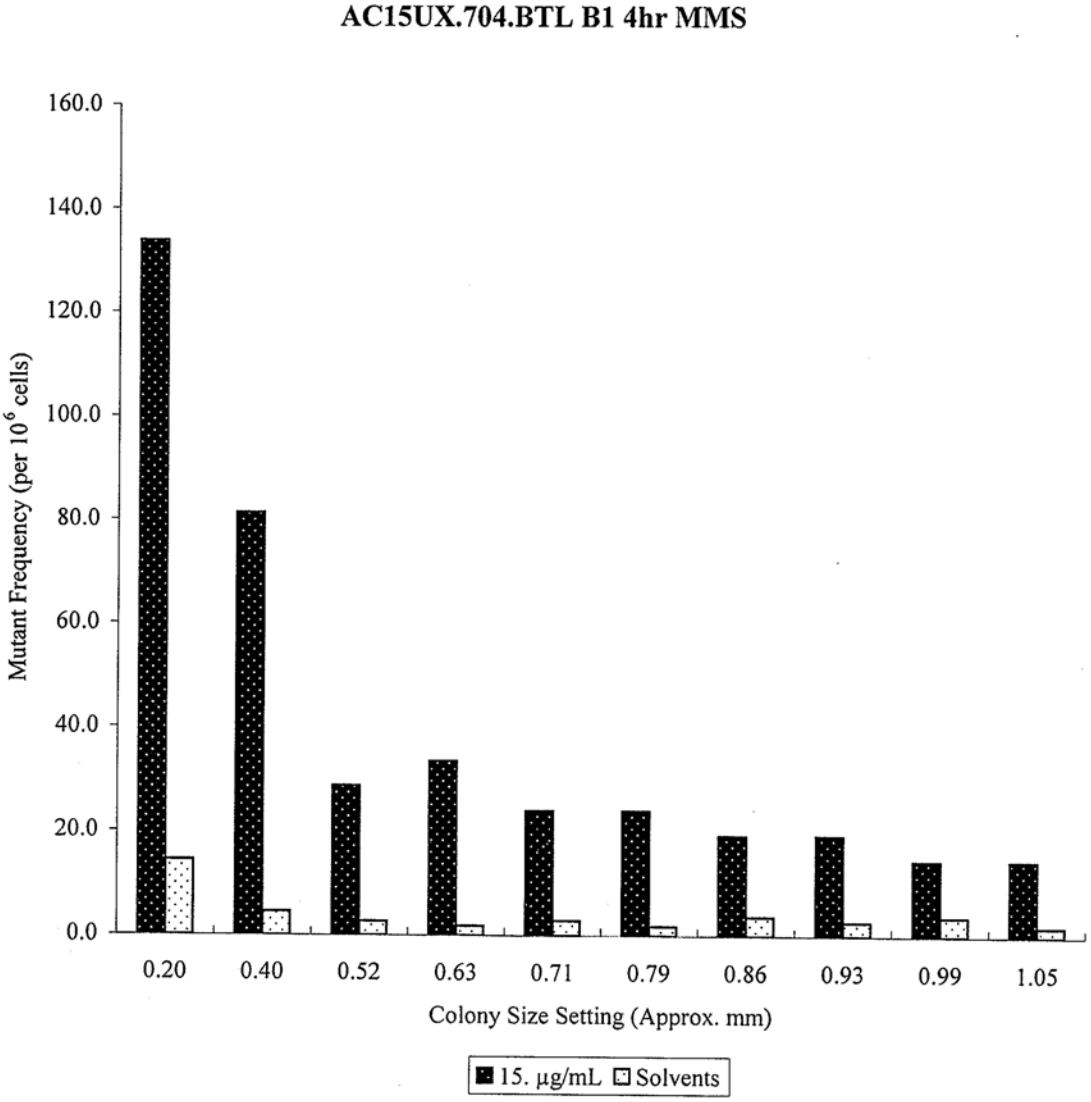


Figure 2
Initial Assay with S9 Activation, 4-Hour Exposure
Colony Size Distribution in the Presence of Metabolic Activation
(Positive Control Compared with Solvent Control)

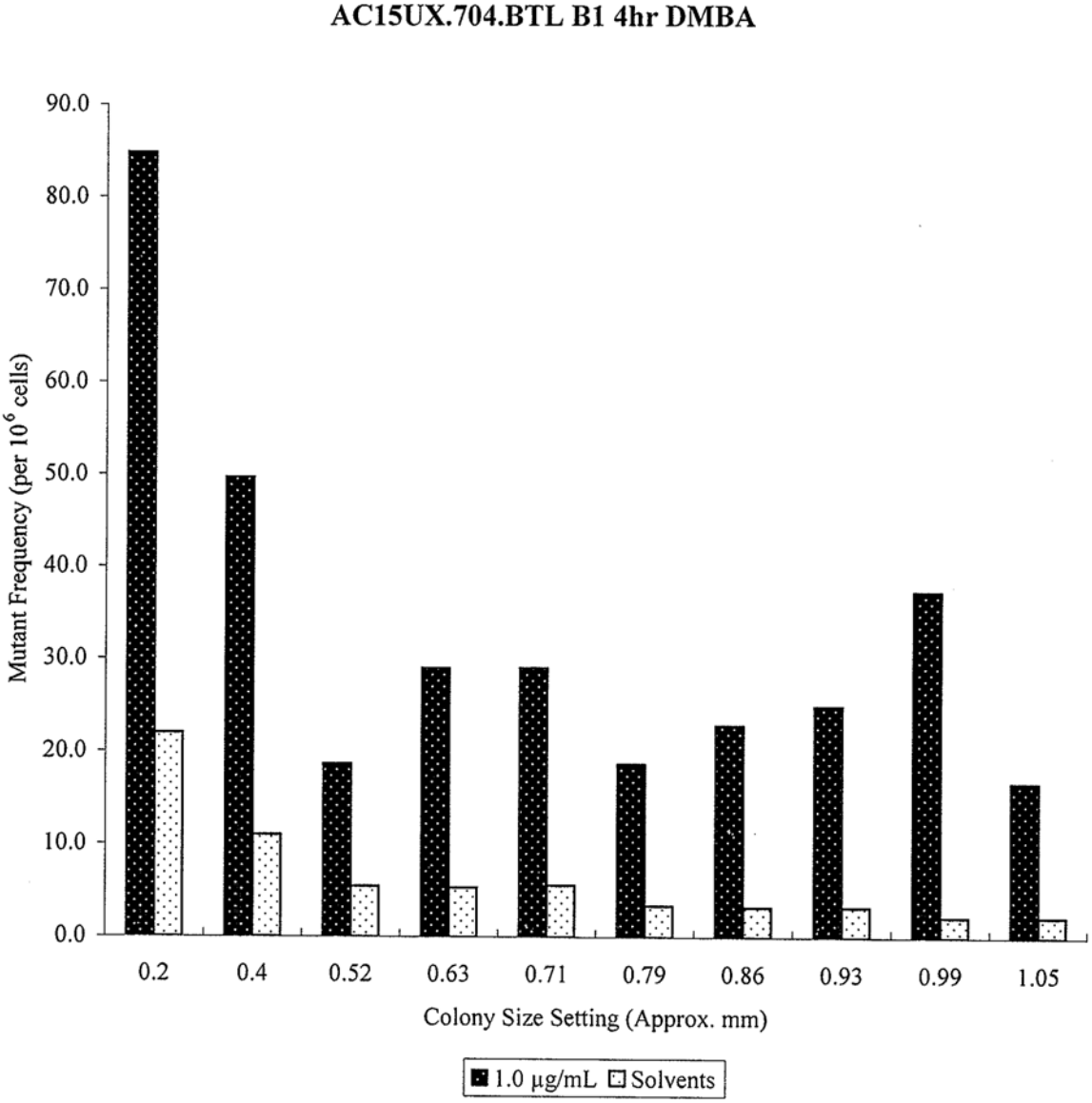
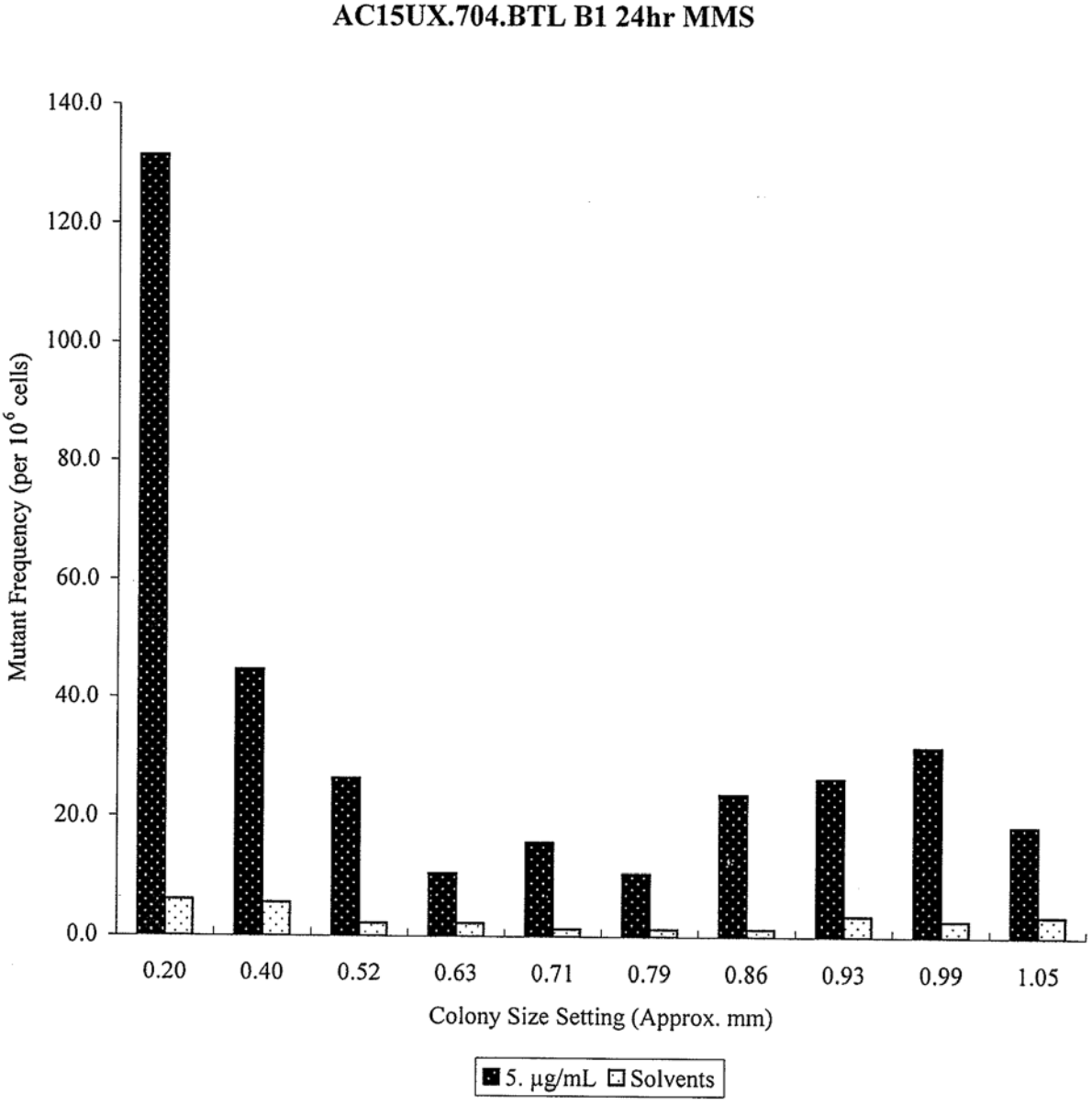


Figure 3
Extended Treatment Assay without S9 Activation, 24-Hour Exposure
Colony Size Distribution in the Presence of Metabolic Activation
(Positive Control Compared with Solvent Control)



APPENDIX A

Historical Control Data

Mouse Lymphoma Historical Control Data

2005-2007

	Non-Activated (4-Hour)			Non-Activated (24-Hour)		
	Solvent Control	15 µg/mL MMS	20 µg/mL MMS	Solvent Control	5.0 µg/mL MMS	7.5 µg/mL MMS
Mean MF	53.1	442.5	624.9	41.6	328.9	522.9
SD	19.1	141.2	192.8	14.1	76.7	126.7
Maximum	116	825	1134	106	651	925
Minimum	25	20	219	22	147	192

	S9-Activated (4-Hour)			
	Solvent Control	0.5 µg/mL DMBA	0.75 µg/mL DMBA	1.0 µg/mL DMBA
Mean MF	57.3	188.9	248.5	322.4
SD	21.9	33.5	43.8	56.5
Maximum	122	270	358	480
Minimum	21	132	167	222

Solvent control: Fischer's medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor. It has been demonstrated that all of the above solvents exhibit the same mutant frequency range.

MMS Methyl methanesulfonate
DMBA Dimethylbenz(a)anthracene
MF Mutant frequency per 10^6 clonable cells
SD Standard deviation

APPENDIX B

Study Protocol

QA Reviewed LPO CPH 20 MAY 08 5/20/08 Init. Date

Received by RA/QA 15-MAY-2008

DuPont-26129
BioReliance Study Number: AC15UX.704.BTL

H-28548: *In Vitro* Mammalian Cell Gene Mutation Test
(L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay)

1.0 PURPOSE

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

2.0 SPONSOR

2.1	Sponsor Name:	E.I. du Pont de Nemours and Company
2.2	Address:	DuPont Haskell Global Centers for Health and Environmental Sciences P.O. Box, 50, 1090 Elkton Road Newark, DE 19714-0050
2.3	Representative:	E. Maria Donner, Ph.D. Phone: 302-366-5251 Fax: 302-451-4531 Email: Maria.Donner@usa.dupont.com
2.4	Work Request No.:	17751
2.5	Haskell Number:	28548
2.6	Service Code :	1537
2.4	Sponsor Report No:	DuPont-26129

3.0 TEST AND CONTROL ARTICLES

3.1	Test Article Name:		H-28548
	Storage Temperature:		Ambient.
	Storage Parameters:		All test articles will be stored in the dark.
	Purity:		An adjustment for 86.6% purity will be made using the correction factor 1.15
	Molecular Weight:		347.09
3.2	Controls:	Negative:	Test article solvent (or vehicle)
		Positive:	Methyl methanesulfonate (MMS) 7,12-dimethylbenz(a)anthracene (DMBA)

3.3 Characterization and Stability of the Test Article

BioReliance will not perform analysis of the test article. The Sponsor will be directly responsible for determination and documentation of the analytical purity, composition and stability of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

Since the in-life portion of this study is less than four weeks in duration, BioReliance will not retain a reserve sample of the test article.

3.5 Residual Test Article and Dosing Preparations

Dosing preparations, excluding those saved for concentration or homogeneity analysis, will be disposed of following administration to the test system. Residual test article will be discarded after finalization of the report.

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: Jane J. Clarke, M.S.
Phone: (301) 610-2219
Fax: (301) 738-2362
E-mail: jane.clarke@bioreliance.com

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 19 May 2008

5.2 Proposed Experimental Completion Date: 09 June 2008

5.3 Proposed Report Date: 23 June 2008

6.0 TEST SYSTEM

L5178Y/TK$^{+/-}$ mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK$^{+/-}$ cells, clone 3.7.2C, were received from Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, NC or American Type Culture Collection, Manassas, VA. 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 and 24 hours in the absence of S9 activation, if the extended treatment assay is necessary. Following a two-day expression period, with daily cell population adjustments, cultures demonstrating 0% to the first concentration showing at least 80% growth inhibition will be cloned, in triplicate, in both complete medium and selective medium containing soft agar. After a 10- to 14-day selection period, the 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 vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system include but are not limited to deionized water (CAS 7732-18-5) and dimethyl sulfoxide (CAS 67-68-5). Ethanol (CAS 64-17-5) is not recommended for 24-hour exposure experiments. The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable/soluble stock concentration, up to 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Based on the molecular weight of the test article, the solvents to be tested and the concentration to be achieved in the assay, alternate stock concentrations may be tested for solubility.

7.2 Concentration 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 not to exceed 5000 µg/mL or 10 mM, whichever is the lower (ICH, 1996; OECD, 1998) unless limited by workability/solubility of the test article. If the molecular weight of the test article is not provided, the highest concentration will be 5000 µg/mL, unless limited by workability/solubility of the test article. 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 F_0P (Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic F-68) or $F_{10}P$ (F_0P supplemented with 10% horse serum and 2 mM L-glutamine) and cultured in suspension for two days post-treatment, with cell concentration adjustment on the first day. After a 24-hour treatment in the absence of S9 activation, cells will be washed with F_0P or $F_{10}P$ and immediately readjusted to 3 x 10^5 cells/mL. Cells will then be cultured in suspension for an additional two days post-treatment with cell concentration adjustment on the first day.

Selection of test article concentration 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 highest test article concentration for the mutation assay will be that concentration exhibiting approximately 100% growth inhibition. For freely soluble, non-toxic test articles, the highest concentration will be 5000 µg/mL or 10 mM, whichever is the lower (ICH, 1996; OECD, 1998). For relatively insoluble test articles, the highest concentration will be the lowest insoluble concentration in treatment medium but not to exceed 5000 µg/mL or 10 mM, whichever is the lower (ICH, 1996; OECD, 1998) irrespective of toxicity. 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

Immediately prior to use, Aroclor 1254-induced rat liver S9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid (or 13.88 mg glucose-6-phosphate), 6 mg NADP, and 0.025 mL S9 homogenate per mL in F_0P. The cofactor mix will be adjusted to pH 7 prior to the addition of S9. Each 10 mL culture will contain 4 mL S9 mix (final S9 concentration of 1%).

7.5 Controls

No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test article and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

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

Results obtained from treatment with these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test article.

Methyl methanesulfonate (MMS) will be used at two concentrations between 1.0 and 20 µg/mL in single cultures as the positive control for the non-activated test system and to determine that the assay is capable of detecting small colonies. For the S9-activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two or three concentrations between 0.5 and 10 µg/mL in single cultures.

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 in 50% conditioned $F_{10}P$ and 50% F_0P.

7.7 Identification of the Test System

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 of dosing solution of test or control article in solvent or solvent alone, F_0P medium or S9 activation mixture with 6 x 10^6 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% CO_2 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 F_0P or $F_{10}P$ and collected by centrifugation. The cells will be resuspended in 20 mL $F_{10}P$, gassed with 5±1% CO_2 in air and cultured in suspension at 37±1°C for two days following treatment. For the 24-hour exposure, the cell population will be adjusted to 3 x 10^5 cells/mL immediately after test article removal. Cell population adjustments to 3 x 10^5 cells/mL will be made at 24 and 48 hours post-treatment for the 4-hr treatment cultures and at 24, 48, and 72 hours post-treatment for the 24-hr treatment cultures.

7.10 Selection of the Mutant Phenotype

For selection of the TK$^{-/-}$ (i.e., trifluorothymidine (TFT)-resistant phenotype), cells will be plated into three replicate dishes at a density of 1 x 10^6 cells/100mm plate in cloning medium containing 0.22% to 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% CO_2 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 (including at least one concentration with between 10% and 20% total growth, if possible). 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 concentration 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 Extended Treatment and/or Confirmatory Assay

Verification of a clear positive response will not be required (OECD Guideline 476; ICH, 1997). For equivocal and negative results without activation, an extended treatment assay will be performed in which cultures are continuously exposed to the test article for 24 hours without S9 activation. A preliminary toxicity assay without S9 activation using a 24-hour continuous treatment may be performed (where appropriate) to select concentrations for the extended treatment assay. The extended treatment assay may be performed concurrently with the initial assay. For equivocal results with S9 activation, a confirmatory assay may be performed using modified concentration levels or study design. For negative results with S9 activation, a confirmatory assay will not be required unless the test article is known to have specific requirements of metabolism.

7.12 Automated Data Collection Systems

The primary computer or electronic systems used for the collection or analysis of data will include but not limited to the following:

The Mouse Lymphoma System (produced in-house), LIMS Labware version 5, Excel 2003 (Microsoft Corporation) and Kaye Lab Watch Monitoring System (Kaye GE).

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The average spontaneous mutant frequency of the solvent (or vehicle) control cultures must be within 35 to 140 TFT-resistant mutants per 10^6 surviving cells. Low spontaneous mutant frequencies, *i.e.*, 20 to 34 mutants per 10^6 surviving cells, are considered acceptable if small colony recovery is demonstrated (Mitchell *et al.*, 1997). The average cloning efficiency of the solvent (or vehicle) controls must be between 65% and 120% and the total suspension growth between 8-32 for the 4-hour exposure and 20 to 180 for the 24-hour exposure (Moore, *et al.*, 2002, 2006, and 2007).

8.2 Positive Controls

The mutant frequency for at least one dose of each positive control must meet the criteria for a positive response. The mutant frequency for at least one dose of one of the positive controls must induce an increase in small colony mutants according to the following criteria: Induced Mutant Frequency (IMF) positive control $\geq 300 \times 10^{-6}$ mutants with 40% small colonies **or** small colony IMF for positive control $\geq 150 \times 10^{-6}$ (Moore, *et al.*, 2002; 2006).

8.3 Test article-Treated Cultures

Cultures treated with a minimum of four concentrations of test article must be attained and their mutant frequencies reported. The highest test article concentration must produce 80% to 90% toxicity (ICH, 1996) unless limited by solubility or the maximum required concentration as described in section 7.2. In the case of a test article with a steep toxicity curve (no concentrations with 10-20% survival), the results may be considered acceptable if a concentration spacing of $\leq$2-fold is used and the highest concentration tested showed <20% survival or total kill (Sofuni *et al.*, 1997). For example, the test is considered acceptable if the highest concentration cloned for mutant selection exhibits >20% survival and the next highest concentration, which is ≤ 2 times the cloned concentration, is too toxic to clone.

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 scientific judgment; however, the following criteria are presented as a guide to interpretation of the data (Moore *et al.*, 2006):

- A result will be considered positive if a concentration-related increase in mutant frequency is observed in the treated cultures and one or more treatment conditions with 10% or greater total growth exhibit mutant frequencies of ≥90 mutants per 10^6 clonable cells over the background level (based on the average mutant frequency of duplicate cultures). If the average solvent control mutant frequency is >90 mutants per 10^6 clonable cells, a doubling of mutant frequency over the background will also be required (Mitchell *et al.*, 1997).

- A result will be considered negative if the treated cultures exhibit mutant frequencies of less than 90 mutants per 10^6 clonable cells over the background level (based on the average mutant frequency of duplicate cultures) and there is no concentration-related increase in mutant frequency.

- There are some situations in which a chemical may be considered negative when there is no culture showing between 10-20% survival: 1) There is no evidence of mutagenicity (e.g. no dose response or increase in mutant frequencies between 45 and 89 mutants per 10^6 above control) in a series of data points within 100% to 20% survival *and* there is at least one negative data point between 20% and 25% survival. 2) There is no evidence of mutagenicity (e.g. no dose response or increase in mutant frequencies between 45 and 89 mutants per 10^6 above control) in a series of data points between 100% to 25% survival *and* there is also a negative data point between 10% and 1% survival (Office of Food Additive Safety, 2001. In this case it is acceptable to count the TFT colonies of cultures exhibiting <10% total growth.

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 for generation and analysis of the data. Unless alternate arrangements are made, the report will be initially issued as a QA-audited draft. After receipt of the draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor or a designated representative, the draft report will be issued as a final report. If all supporting analytical documents have not been provided to BioReliance, the report will be written based on those that are provided to BioReliance.

The report will include:

- Test article: 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, CO_2 concentration, concentration of test article, 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
- Concentration-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 concentration level of the test article (the highest positive concentration)
- Positive and solvent control historical data
- Statement of Compliance
- Quality Assurance Statement

If an electronic copy of the protocol, the report or another study document is provided by BioReliance, the executed paper document is considered the official master document. If there is a discrepancy between an electronic copy and the corresponding master document, the master document will be considered the official document. Six months after issuance of the draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor or a designated representative, the draft report will be issued as a final report. If all supporting analytical documents have not been provided to BioReliance, the report will be written based on those that are provided to BioReliance.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports, generated by BioReliance, will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance Quality Assurance unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied onto electronic media and the electronic copy will be retained in the BioReliance archives for a minimum of 10 years.

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), February 1998, 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, the 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, with the US EPA Health Effects Test Guidelines, OPPTS 870.5300 (1998), and with EC Commission Directive 2000/32/EC, Annex 4E No. L136.

The following Good Laboratory Practices (GLP) regulations will be followed at BioReliance as requested by the Sponsor.

- US FDA Good Laboratory Practices 21 CFR Part 58
- OECD Principles of Good Laboratory Practice (C(97)186/Final)
- US EPA GLP Standards 40 CFR 792

For the study, an in-process phase, the raw data, and report(s) will be inspected per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA Statement will be included in the final report. This statement will list the study-specific phases inspected at BioReliance, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP Compliance Statement will be included in the final report. This statement will cite the GLP regulations with which this study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test article or its mixtures.

Raw data, the protocol and reports generated at locations other than BioReliance will or will not be QA audited per the contractual arrangements between that site and the Sponsor.

Alterations of this protocol may be made as the study progresses. All protocol procedural modifications and rationale for the change(s) will be documented, signed, dated and approved by the Study Director, BioReliance QA and the Sponsor. All applicable protocol amendments will be delivered to the Sponsor via mail, electronic file transfer or fax transmission, as well as internally at the Test Facility, on or as close as possible to the effective date of the amendment.

Deviations from the protocol and/or BioReliance SOPs will be documented in a deviation report or a note to file will be generated. The deviation report will be signed by the Study Director, Test Facility Management and BioReliance QA.

13.0 REFERENCES

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14.0 APPROVAL

14.1 Sponsor Approval

E. Maria Donner, Ph.D.
Sponsor Representative

13-MAY-2008
Date

DuPont-26129
BioReliance Study Number: AC15UX.704.BTL

14.2 Study Director and Test Facility Management Approvals

15 May 2008
Date

Jane J. Clarke, M.S.
BioReliance Study Director

15 May 2008
Date

BioReliance Study Management

APPENDIX C

Certificate of Analysis

E. I. du Pont de Nemours and Company
Wilmington, DE 19898
USA

CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28548
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	84%
Other Components	Water – 12.7% Perfluorooctanoic acid – 150 ppm
Date of Analysis	June 13, 2008
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-25455
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Approver:

Peter A. Bloxham, Ph.D.
Senior Research Chemist

18-JUNE-2008
Date