TRADE SECRET

Study Title

H-25171: Bacterial Reverse Mutation Test with an Independent Repeat Assay

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Study Completion Date

11 February 2002

Performing Laboratory

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Performing Laboratory Study Number

AA52XL.502001.BTL

Work Request Number

[redacted]

Service Code

[redacted]

STATEMENT OF COMPLIANCE

Study No. AA52XL.502001.BTL was conducted in compliance with the U.S. FDA GLP Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160, and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test and control articles have not been determined by the testing facility. The control articles have been characterized as per the Certificates of Analysis on file with the testing facility.

The stability of the test and control articles has not been determined by the testing facility.

Analyses to determine the uniformity (as applicable), or concentration of the test and control mixtures were not performed by the testing facility. The Sponsor has indicated that they have not performed these analyses on the test substance mixtures.

The stability of the test and control articles in the test and control mixtures, respectively, has not been determined by the testing facility. The Sponsor has indicated that they have not performed stability analysis on the test substance mixtures.

Valentine O. Wagner, III — 11 Feb 2002

Valentine O. Wagner, III, M.S.
Study Director

Date

Rick — 11 Feb 2002

BioReliance Study Management

Date

Quality Assurance Statement

Study Title: H-25171: BACTERIAL REVERSE MUTATION ASSAY WITH AN INDEPENDENT REPEAT ASSAY

Study Number: AA52XL.502001.BTL

Study Director: Valentine O. Wagner, III, M.S.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is 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	06-Dec-01 - 06-Dec-01 To Study Dir 06-Dec-01 To Mgmt 06-Dec-01
Phase	Protocol Review
Inspect On	24-Dec-01 - 24-Dec-01 To Study Dir 24-Dec-01 To Mgmt 26-Dec-01
Phase	Scoring the plates
Inspect On	03-Feb-02 - 03-Feb-02 To Study Dir 04-Feb-02 To Mgmt 07-Feb-02
Phase	Draft Report
Inspect On	11-Feb-02 - 11-Feb-02 To Study Dir 11-Feb-02 To Mgmt 11-Feb-02
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.

Becky D. Schreckengost — 11 Feb 2002

Becky D. Schreckengost, B.S.
QUALITY ASSURANCE

DATE

CERTIFICATION

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

Issued by Study Director: Valentine O. Wagner, III — 11 Feb 2002
Valentine O. Wagner III, M.S. — Date

Approved by Study Monitor: Maria Donner — 08-Feb-2002
Maria Donner, Ph.D. — Date
Senior Research Scientist

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STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes:
- [redacted]
- H-25171
- [redacted]

Submitter's Notebook Number(s): [redacted]

Haskell Number: 25171

CAS Registry Number: [redacted]

Composition: [redacted]

Purity: [redacted]

Known Impurities: [redacted]

Physical Characteristics: [redacted]

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

Sponsor: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: December 05, 2001 / (see report cover page)

In-Life Initiated/Completed: December 20, 2001 / January 22, 2002

SUMMARY

The test substance, H-25171, was tested in the Bacterial Reverse Mutation Test with an Independent Repeat Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The test was performed in two phases, using the plate incorporation method. The first phase, the preliminary toxicity test, was used to establish the dose-range for the mutagenicity test. The second phase, the mutagenicity test (initial and independent repeat tests), was used to evaluate the mutagenic potential of the test substance.

Tetrahydrofuran (THF) was selected as the solvent of choice based on compatibility with the target cells and solubility of the test substance. The test substance was soluble but cloudy in tetrahydrofuran (THF) at a maximum concentration of approximately 200 mg/mL.

In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 200 mg/mL and a 25 µL plating aliquot. The test substance was a workable solution from 40 to 200 mg/mL and a soluble and clear solution from 0.27 to 27 mg/mL. Dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Precipitate was observed beginning at 100 µg per plate. No appreciable toxicity was observed. Based on the findings of the toxicity test, the maximum dose plated in the mutagenicity test was 5000 µg per plate.

In the mutagenicity test, no positive mutagenic response was observed. The dose levels tested were 15, 50, 150, 500, 1500 and 5000 µg per plate. Precipitate was observed beginning at 500 µg per plate. No appreciable toxicity was observed.

The results of the Bacterial Reverse Mutation Test with an Independent Repeat Assay indicate that, under the conditions of this study, H-25171 did not cause a positive mutagenic response in either the presence or absence of Aroclor-induced rat liver S9.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. A copy of the protocol is included in Appendix B.

This study was conducted in compliance with the testing guidelines of the ICH (1996 and 1997) and OECD (1998).

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, H-25171, was received by BioReliance on 03 December 2001 and was assigned the code number AA52XL. The test substance was characterized by the Sponsor as a [redacted] that should be stored at ambient temperature. An expiration date of 26 November 2004 was provided. Upon receipt, the test substance was described as a [redacted] and was stored at room temperature, protected from light and moisture. The identity, strength, purity, composition or other characteristics to define the test substance and the stability of the test substance have been determined by the Sponsor. The Sponsor indicated that BioReliance would not receive copies of these reports.

The vehicle used to deliver H-25171 to the test system was tetrahydrofuran (THF, CAS# 109-99-9), Gold Label, 99.9%, purchased from Aldrich Chemical Company. Test substance dilutions were prepared immediately before use and delivered to the test system under yellow light. To enhance the workability of the test substance, the top concentration was sonicated in a screw-cap tube for approximately 15 minutes at 50°C. Subsequent dilutions were maintained at 50°C in screw-cap tubes during dosing.

Positive controls plated concurrently with the mutagenicity tests are listed below. All positive controls were diluted with dimethyl sulfoxide (DMSO) except sodium azide, which was diluted with water. All subdivided solutions of positive control were stored at –5 to –30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
All *Salmonella* Strains	Rat	2-aminoanthracene (Aldrich Chemical Co., Inc.) Lot No. 09106PS Exp. Date 14-Sep-2005 CAS No. 613-13-8 Purity >95%	1.0
WP2 *uvr*A			10

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98	None	2-nitrofluorene (Aldrich Chemical Co., Inc.) Lot No. 08707HS Exp. Date 08-Mar-2006 CAS No. 607-57-8 Purity >98%	1.0
TA100, TA1535		Sodium azide (Sigma Chemical Co.) Lot No. 098H0169 Exp. Date 05-Jan-2004 CAS No. 26628-22-8 Purity >99%	1.0
TA1537		9-aminoacridine (Sigma Chemical Co.) Lot No. 106F06681 Exp. Date 18-Nov-2004 CAS No. 90-45-9 Purity >98%	75
WP2 *uvr*A		Methyl methanesulfonate (Aldrich Chemical Co., Inc.) Lot No. 15526AO Exp. Date 28-Nov-2003 CAS No. 66-27-3 Purity >99%	1,000

To confirm the sterility of the test substance, the highest test substance dose level used in the mutagenicity tests was plated on selective agar with an aliquot volume equal to that used in the test. These plates were incubated under the same conditions as the test.

MATERIALS AND METHODS

For submission to Japanese regulatory agencies, additional information is included in Appendix C.

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976). *Salmonella* tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley and *E. coli* tester strains were received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to base-pair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count tests on nutrient agar plates, and the data is on file but not presented in this report. The study was conducted to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), adopted July 1997 (published February 1998) and with the International Conference on Harmonisation of Technical Requirements of Registration of Pharmaceuticals for Human Use (1996 and 1997).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at -70°C or colder until used. Each bulk preparation of S9 was tested for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM ß-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Solubility Test

A solubility test was conducted to select the vehicle. The test was conducted using water, dimethyl sulfoxide (DMSO), ethanol (EtOH), acetone, tetrahydrofuran (THF), acetonitrile, propylene glycol and polyethylene glycol. The test substance was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 50 mg/mL for aqueous solvents and 500 mg/mL for organic solvents.

Preliminary Toxicity Test

The preliminary toxicity test was used to establish the dose-range over which the test substance would be tested. Vehicle and ten dose levels of the test substance were plated, one plate per dose, with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9. Dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate.

Mutagenicity Test

The mutagenicity test (initial and independent repeat tests) was used to evaluate the mutagenic potential of the test substance. Six dose levels of test substance (15, 50, 150, 500, 1500 and 5000 µg per plate) along with appropriate vehicle and positive controls were plated with TA98, TA100, TA1535, TA1537 and WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. All dose levels of test substance, vehicle controls and positive controls were plated in triplicate.

Plating and Scoring Procedures

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and

reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test substance, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's Standard Operating Procedures.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain and 25 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted (less than 10 days).

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance precipitate.

Code	Description	Characteristics
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., less than 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., more than 3 particles on a plate with 30 revertants). These plates are counted manually.

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity.

Evaluation of Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance. Data sets for tester strains TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value. Data sets for tester strains TA98, TA100 and WP2 *uvr*A were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity test to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 0.3×10^9 cells/mL. The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels is required to evaluate test data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of

revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A moderate reduction in the background lawn (background code of 3, 4 or 5). A copy of the Historical Negative and Positive Control Values is included in Appendix A.

Archives

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure [redacted] 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. Unused dosing solutions were disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

Deviations

No known deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Tetrahydrofuran (THF) was selected as the solvent of choice based on compatibility with the target cells and solubility of the test substance. The test substance was soluble but cloudy in tetrahydrofuran (THF) at a maximum concentration of approximately 200 mg/mL.

Preliminary Toxicity Test

The results of the preliminary toxicity test are presented in Tables 1 through 4. These data were generated in Experiment A1. In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 200 mg/mL and a 25 µL plating aliquot. The test substance was a workable solution from 40 to 200 mg/mL and a soluble and clear solution from 0.27 to 27 mg/mL. Dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Precipitate was observed beginning at 100 µg per plate. No appreciable toxicity was observed. Due to tester strain culture contamination, tester strain TA100 in the presence and absence of S9 activation was only evaluated for precipitate and toxicity. Based on the findings of the toxicity test, the maximum dose plated in the mutagenicity test was 5000 µg per plate.

Mutagenicity Test

The results of the mutagenicity test are presented in Tables 5 through 24 and summarized in Tables 25 and 26. These data were generated in Experiments B1 and B2. Dose levels tested were 15, 50, 150, 500, 1500 and 5000 µg per plate. Precipitate was observed beginning at 500 µg per plate. No appreciable toxicity was observed. For submission to Japanese regulatory agencies, pertinent additional information is included in Appendix C.

In Experiment B1 (Initial Mutagenicity Test), no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

In Experiment B2 (Independent Repeat Test), no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Test with an Independent Repeat Assay indicate that, under the conditions of this study, H-25171 did not cause a positive mutagenic response in either the presence or absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

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.

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Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

Bacterial Mutation Test
Preliminary Toxicity Test

Table 1

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Experiment No. : A1
Strain : TA98
Date Plated : 20 Dec 2001
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	23	1	23	1
6.7	33	1	14	1
10	22	1	19	1
33	24	1	16	1
67	34	1	14	1
100	29	1NP	13	1NP
333	23	1NP	13	1NP
667	22	1NP	17	1NP
1000	24	1NP	15	1NP
3333	22	1NP	12	1NP
5000	30	1NP	15	1NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 2

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Experiment No. : A1
Strain : TA1535
Date Plated : 20 Dec 2001
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	15	1	22	1
6.7	17	1	19	1
10	10	1	13	1
33	11	1	18	1
67	17	1	15	1
100	9	1NP	11	1NP
333	12	1NP	12	1NP
667	18	1NP	19	1NP
1000	15	1NP	C	
3333	8	1NP	12	1NP
5000	10	1NP	20	1NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
C=Contaminated

Bacterial Mutation Test
Preliminary Toxicity Test

Table 3

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Experiment No. : A1
Strain : TA1537
Date Plated : 20 Dec 2001
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	6	1	7	1
6.7	10	1	5	1
10	7	1	12	1
33	7	1	7	1
67	5	1	6	1
100	11	1NP	6	1NP
333	10	1NP	8	1NP
667	10	1NP	12	1NP
1000	4	1NP	8	1NP
3333	9	1NP	7	1NP
5000	10	1NP	6	1NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Preliminary Toxicity Test

Table 4

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Experiment No. : A1
Strain : WP2 uvrA
Date Plated : 20 Dec 2001
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Test Substance Concentration µg per plate	With S9 Activation Revertants per plate	With S9 Activation Background Lawn	Without S9 Activation Revertants per plate	Without S9 Activation Background Lawn
Vehicle	22	1	18	1
6.7	22	1	22	1
10	29	1	14	1
33	13	1	17	1
67	20	1	15	1
100	15	1NP	21	1NP
333	19	1NP	13	1NP
667	21	1NP	17	1NP
1000	25	1NP	31	1NP
3333	18	1NP	11	1NP
5000	26	1NP	18	1NP

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 5

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 2.7 X 10^8
Liver Microsomes : None Date Plated : 4 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	15	1		
	02	14	1		
	03	20	1	16	3
15	01	22	1		
	02	10	1		
	03	14	1	15	6
50	01	13	1		
	02	18	1		
	03	12	1	14	3
150	01	16	1		
	02	13	1		
	03	10	1	13	3
500	01	13	1NP		
	02	15	1NP		
	03	10	1NP	13	3
1500	01	14	1NP		
	02	14	1NP		
	03	18	1NP	15	2
5000	01	10	1NP		
	02	12	1IP		
	03	12	1IP	11	1
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	217	1		
	02	184	1		
	03	232	1	211	25

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 6

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 2.7 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 4 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	33	1		
	02	20	1		
	03	29	1	27	7
15	01	26	1		
	02	26	1		
	03	23	1	25	2
50	01	17	1		
	02	25	1		
	03	17	1	20	5
150	01	28	1		
	02	32	1		
	03	21	1	27	6
500	01	23	1NP		
	02	25	1NP		
	03	20	1NP	23	3
1500	01	35	1NP		
	02	38	1NP		
	03	25	1NP	33	7
5000	01	18	1IP		
	02	25	1IP		
	03	20	1IP	21	4
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	480	1		
	02	483	1		
	03	338	1	434	83

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 7

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 1.1 X 10^8
Liver Microsomes : None Date Plated : 4 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	223	1		
	02	190	1		
	03	182	1	198	22
15	01	168	1		
	02	163	1		
	03	179	1	170	8
50	01	166	1		
	02	191	1		
	03	188	1	182	14
150	01	199	1		
	02	179	1		
	03	150	1	176	25
500	01	140	1NP		
	02	145	1NP		
	03	C		143	4
1500	01	179	1NP		
	02	202	1NP		
	03	143	1NP	175	30
5000	01	163	1NP		
	02	206	1NP		
	03	192	1NP	187	22
Positive Control sodium azide 1.0 µg per plate					
	01	672	1		
	02	618	1		
	03	851	1	714	122

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
C=Contaminated

Bacterial Mutation Test

Table 8

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 1.1 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 4 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	151	1		
	02	163	1		
	03	204	1	173	28
15	01	210	1		
	02	172	1		
	03	159	1	180	27
50	01	190	1		
	02	170	1		
	03	178	1	179	10
150	01	198	1		
	02	171	1		
	03	185	1	185	14
500	01	171	1NP		
	02	195	1NP		
	03	165	1NP	177	16
1500	01	214	1NP		
	02	187	1NP		
	03	177	1NP	193	19
5000	01	195	1NP		
	02	183	1NP		
	03	226	1NP	201	22
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	877	1		
	02	850	1		
	03	722	1	816	83

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 9

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1535
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL
Experiment No : B1
Cells Seeded : 2.6 X 10^8
Date Plated : 4 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	15	1		
	03	19	1	15	4
15	01	15	1		
	02	16	1		
	03	15	1	15	1
50	01	12	1		
	02	17	1		
	03	11	1	13	3
150	01	23	1		
	02	19	1		
	03	15	1	19	4
500	01	13	1NP		
	02	22	1NP		
	03	15	1NP	17	5
1500	01	12	1NP		
	02	15	1NP		
	03	8	1NP	12	4
5000	01	18	1IP		
	02	12	1IP		
	03	19	1NP	16	4
Positive Control sodium azide 1.0 µg per plate					
	01	402	1		
	02	476	1		
	03	414	1	431	40

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 10

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL
Experiment No : B1
Cells Seeded : 2.6 X 10^8
Date Plated : 4 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	15	1		
	02	21	1		
	03	25	1	20	5
15	01	26	1		
	02	24	1		
	03	21	1	24	3
50	01	12	1		
	02	16	1		
	03	16	1	15	2
150	01	20	1		
	02	10	1		
	03	14	1	15	5
500	01	14	1NP		
	02	20	1NP		
	03	15	1NP	16	3
1500	01	14	1NP		
	02	14	1NP		
	03	8	1NP	12	3
5000	01	23	1NP		
	02	13	1NP		
	03	21	1NP	19	5
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	173	1		
	02	128	1		
	03	146	1	149	23

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 11

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1537
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B1
Cells Seeded : 1.3 X 10^8
Date Plated : 4 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	3	1		
	02	7	1		
	03	4	1	5	2
15	01	5	1		
	02	8	1		
	03	1	1	5	4
50	01	3	1		
	02	8	1		
	03	3	1	5	3
150	01	11	1		
	02	5	1		
	03	6	1	7	3
500	01	4	1NP		
	02	5	1NP		
	03	6	1NP	5	1
1500	01	5	1NP		
	02	3	1NP		
	03	5	1NP	4	1
5000	01	9	1IP		
	02	8	1IP		
	03	3	1IP	7	3
Positive Control 9-aminoacridine 75 µg per plate					
	01	543	1		
	02	352	1		
	03	616	1	504	136

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 12

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1537
Liver Microsomes : Rat liver S9
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B1
Cells Seeded : 1.3 X 10^8
Date Plated : 4 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	8	1		
	02	13	1		
	03	8	1	10	3
15	01	3	1		
	02	13	1		
	03	8	1	8	5
50	01	7	1		
	02	6	1		
	03	10	1	8	2
150	01	9	1		
	02	7	1		
	03	10	1	9	2
500	01	4	1NP		
	02	8	1NP		
	03	9	1NP	7	3
1500	01	6	1NP		
	02	8	1NP		
	03	4	1NP	6	2
5000	01	9	1IP		
	02	6	1IP		
	03	5	1NP	7	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	96	1		
	02	103	1		
	03	84	1	94	10

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 13

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : WP2 *uvrA*
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL
Experiment No : B1
Cells Seeded : 2.5 X 10^8
Date Plated : 4 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	12	1		
	03	15	1	14	2
15	01	17	1		
	02	14	1		
	03	13	1	15	2
50	01	16	1		
	02	12	1		
	03	11	1	13	3
150	01	16	1		
	02	14	1		
	03	10	1	13	3
500	01	11	1NP		
	02	10	1NP		
	03	17	1NP	13	4
1500	01	13	1NP		
	02	18	1NP		
	03	12	1NP	14	3
5000	01	11	1IP		
	02	10	1IP		
	03	8	1IP	10	2
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	89	1		
	02	92	1		
	03	97	1	93	4

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 14

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1
Strain : WP2 uvrA Cells Seeded : 2.5 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 4 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	16	1		
	02	19	1		
	03	12	1	16	4
15	01	18	1		
	02	16	1		
	03	11	1	15	4
50	01	21	1		
	02	16	1		
	03	14	1	17	4
150	01	10	1		
	02	16	1		
	03	13	1	13	3
500	01	14	1NP		
	02	10	1NP		
	03	11	1NP	12	2
1500	01	15	1NP		
	02	16	1NP		
	03	13	1NP	15	2
5000	01	14	1IP		
	02	17	1NP		
	03	9	1NP	13	4
Positive Control 2-aminoanthracene 10 µg per plate					
	01	965	1		
	02	584	1		
	03	749	1	766	191

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 15

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA98
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B2
Cells Seeded : 1.0 X 10^8
Date Plated : 15 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	37	1		
	02	24	1		
	03	21	1	27	9
15	01	24	1		
	02	16	1		
	03	29	1	23	7
50	01	29	1		
	02	16	1		
	03	27	1	24	7
150	01	29	1		
	02	27	1		
	03	27	1	28	1
500	01	19	1NP		
	02	15	1NP		
	03	15	1NP	16	2
1500	01	24	1IP		
	02	32	1IP		
	03	13	1IP	23	10
5000	01	37	1IP		
	02	31	1IP		
	03	14	1IP	27	12
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	158	1		
	02	98	1		
	03	171	1	142	39

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 16

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B2
Cells Seeded : 1.0 X 10^8
Date Plated : 15 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	32	1		
	02	16	1		
	03	26	1	25	8
15	01	28	1		
	02	26	1		
	03	31	1	28	3
50	01	47	1		
	02	35	1		
	03	18	1	33	15
150	01	49	1		
	02	35	1		
	03	44	1	43	7
500	01	38	1NP		
	02	35	1NP		
	03	20	1NP	31	10
1500	01	34	1NP		
	02	43	1NP		
	03	27	1NP	35	8
5000	01	24	1IP		
	02	37	1IP		
	03	32	1IP	31	7
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	631	1		
	02	718	1		
	03	852	1	734	111

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 17

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA100
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B2
Cells Seeded : 0.9 X 10^8
Date Plated : 15 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	140	1		
	02	125	1		
	03	100	1	122	20
15	01	154	1		
	02	150	1		
	03	178	1	161	15
50	01	156	1		
	02	C			
	03	178	1	167	16
150	01	148	1		
	02	126	1		
	03	167	1	147	21
500	01	178	1NP		
	02	127	1NP		
	03	156	1NP	154	26
1500	01	152	1NP		
	02	131	1NP		
	03	132	1NP	138	12
5000	01	104	1IP		
	02	136	1IP		
	03	C		120	23
Positive Control sodium azide 1.0 µg per plate					
	01	347	1		
	02	364	1		
	03	387	1	366	20

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
C=Contaminated

Bacterial Mutation Test

Table 18

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2
Strain : TA100 Cells Seeded : 0.9 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 15 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	171	1		
	02	152	1		
	03	193	1	172	21
15	01	207	1		
	02	190	1		
	03	156	1	184	26
50	01	182	1		
	02	169	1		
	03	181	1	177	7
150	01	166	1		
	02	150	1		
	03	161	1	159	8
500	01	142	1NP		
	02	168	1NP		
	03	144	1NP	151	14
1500	01	151	1IP		
	02	171	1IP		
	03	116	1IP	146	28
5000	01	187	1IP		
	02	169	1IP		
	03	156	1IP	171	16
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	837	1		
	02	714	1		
	03	664	1	738	89

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 19

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2
Strain : TA1535 Cells Seeded : 0.9 X 10^8
Liver Microsomes : None Date Plated : 15 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	8	1		
	03	12	1	11	3
15	01	16	1		
	02	10	1		
	03	11	1	12	3
50	01	13	1		
	02	11	1		
	03	9	1	11	2
150	01	11	1		
	02	16	1		
	03	11	1	13	3
500	01	9	1IP		
	02	8	1IP		
	03	11	1NP	9	2
1500	01	12	1IP		
	02	18	1IP		
	03	11	1IP	14	4
5000	01	13	1IP		
	02	13	1IP		
	03	16	1IP	14	2
Positive Control sodium azide 1.0 µg per plate					
	01	179	1		
	02	216	1		
	03	185	1	193	20

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 20

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2
Strain : TA1535 Cells Seeded : 0.9 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 15 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	8	1		
	03	11	1	11	3
15	01	NC			
	02	14	1		
	03	9	1	12	4
50	01	12	1		
	02	10	1		
	03	19	1	14	5
150	01	13	1		
	02	13	1		
	03	13	1	13	0
500	01	14	1NP		
	02	18	1NP		
	03	10	1NP	14	4
1500	01	10	1IP		
	02	17	1IP		
	03	10	1IP	12	4
5000	01	19	1IP		
	02	8	1IP		
	03	11	1IP	13	6
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	142	1		
	02	106	1		
	03	88	1	112	27

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
NC=No count due to procedural error in which the plate did not receive an aliquot of tester strain

Bacterial Mutation Test

Table 21

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1537
Liver Microsomes : None
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL
Experiment No : B2
Cells Seeded : 0.5 X 10^8
Date Plated : 15 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	5	1		
	02	2	1		
	03	4	1	4	2
15	01	6	1		
	02	6	1		
	03	4	1	5	1
50	01	7	1		
	02	7	1		
	03	4	1	6	2
150	01	5	1		
	02	2	1		
	03	6	1	4	2
500	01	7	1NP		
	02	4	1NP		
	03	4	1NP	5	2
1500	01	8	1IP		
	02	4	1IP		
	03	3	1IP	5	3
5000	01	7	1IP		
	02	7	1IP		
	03	4	1IP	6	2
Positive Control 9-aminoacridine 75 µg per plate					
	01	663	1		
	02	342	1		
	03	641	1	549	179

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 22

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL
Strain : TA1537
Liver Microsomes : Rat liver S9
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Experiment No : B2
Cells Seeded : 0.5 X 10^8
Date Plated : 15 Jan 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	5	1		
	02	6	1		
	03	8	1	6	2
15	01	4	1		
	02	3	1		
	03	6	1	4	2
50	01	4	1		
	02	5	1		
	03	12	1	7	4
150	01	8	1		
	02	9	1		
	03	6	1	8	2
500	01	5	1NP		
	02	3	1NP		
	03	7	1NP	5	2
1500	01	3	1IP		
	02	6	1IP		
	03	6	1IP	5	2
5000	01	3	1IP		
	02	5	1IP		
	03	7	1IP	5	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	96	1		
	02	70	1		
	03	74	1	80	14

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test

Table 23

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2
Strain : WP2 *uvrA* Cells Seeded : 2.1 X 10^8
Liver Microsomes : None Date Plated : 15 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	11	1		
	03	10	1	11	1
15	01	10	1		
	02	7	1		
	03	9	1	9	2
50	01	15	1		
	02	12	1		
	03	7	1	11	4
150	01	4	1		
	02	9	1		
	03	11	1	8	4
500	01	7	1NP		
	02	17	1NP		
	03	7	1NP	10	6
1500	01	C			
	02	12	1IP		
	03	13	1IP	13	1
5000	01	13	1IP		
	02	14	1IP		
	03	14	1IP	14	1
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	140	1		
	02	81	1		
	03	138	1	120	34

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
C=Contaminated

Bacterial Mutation Test

Table 24

Test Substance Id: H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2
Strain : WP2 uvrA Cells Seeded : 2.1 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 15 Jan 2002
Vehicle : tetrahydrofuran
Plating Aliquot : 25 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	16	1		
	02	22	1		
	03	10	1	16	6
15	01	20	1		
	02	13	1		
	03	15	1	16	4
50	01	13	1		
	02	12	1		
	03	15	1	13	2
150	01	17	1		
	02	16	1		
	03	10	1	14	4
500	01	13	1NP		
	02	8	1NP		
	03	16	1NP	12	4
1500	01	14	1IP		
	02	12	1IP		
	03	13	1IP	13	1
5000	01	13	1IP		
	02	7	1IP		
	03	12	1IP	11	3
Positive Control 2-aminoanthracene 10 µg per plate					
	01	816	1		
	02	1128	1		
	03	938	1	961	157

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Moderately reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Test
Summary of Results

Table 25

Test Substance Id : H-25171
Study Number : AA52XL.502001.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	16 ± 3	198 ± 22	15 ± 4	5 ± 2	14 ± 2
15	15 ± 6	170 ± 8	15 ± 1	5 ± 4	15 ± 2
50	14 ± 3	182 ± 14	13 ± 3	5 ± 3	13 ± 3
150	13 ± 3	176 ± 25	19 ± 4	7 ± 3	13 ± 3
500	13 ± 3	143 ± 4	17 ± 5	5 ± 1	13 ± 4
1500	15 ± 2	175 ± 30	12 ± 4	4 ± 1	14 ± 3
5000	11 ± 1	187 ± 22	16 ± 4	7 ± 3	10 ± 2
Positive	211 ± 25	714 ± 122	431 ± 40	504 ± 136	93 ± 4

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	27 ± 7	173 ± 28	20 ± 5	10 ± 3	16 ± 4
15	25 ± 2	180 ± 27	24 ± 3	8 ± 5	15 ± 4
50	20 ± 5	179 ± 10	15 ± 2	8 ± 2	17 ± 4
150	27 ± 6	185 ± 14	15 ± 5	9 ± 2	13 ± 3
500	23 ± 3	177 ± 16	16 ± 3	7 ± 3	12 ± 2
1500	33 ± 7	193 ± 19	12 ± 3	6 ± 2	15 ± 2
5000	21 ± 4	201 ± 22	19 ± 5	7 ± 2	13 ± 4
Positive	434 ± 83	816 ± 83	149 ± 23	94 ± 10	766 ± 191

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 25 µL

Bacterial Mutation Test
Summary of Results

Table 26

Test Substance Id : H-25171
Study Number : AA52XL.502001.BTL Experiment No : B2

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	27 ± 9	122 ± 20	11 ± 3	4 ± 2	11 ± 1
15	23 ± 7	161 ± 15	12 ± 3	5 ± 1	9 ± 2
50	24 ± 7	167 ± 16	11 ± 2	6 ± 2	11 ± 4
150	28 ± 1	147 ± 21	13 ± 3	4 ± 2	8 ± 4
500	16 ± 2	154 ± 26	9 ± 2	5 ± 2	10 ± 6
1500	23 ± 10	138 ± 12	14 ± 4	5 ± 3	13 ± 1
5000	27 ± 12	120 ± 23	14 ± 2	6 ± 2	14 ± 1
Positive	142 ± 39	366 ± 20	193 ± 20	549 ± 179	120 ± 34

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	25 ± 8	172 ± 21	11 ± 3	6 ± 2	16 ± 6
15	28 ± 3	184 ± 26	12 ± 4	4 ± 2	16 ± 4
50	33 ± 15	177 ± 7	14 ± 5	7 ± 4	13 ± 2
150	43 ± 7	159 ± 8	13 ± 0	8 ± 2	14 ± 4
500	31 ± 10	151 ± 14	14 ± 4	5 ± 2	12 ± 4
1500	35 ± 8	146 ± 28	12 ± 4	5 ± 2	13 ± 1
5000	31 ± 7	171 ± 16	13 ± 6	5 ± 2	11 ± 3
Positive	734 ± 111	738 ± 89	112 ± 27	80 ± 14	961 ± 157

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 25 µL

APPENDIX A

Historical Control Data

Historical Negative and Positive Control Values
1998 – 2000

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	16	7	4	59	21	7	7	58
	Pos	425	206	21	1536	592	322	56	2454
TA100	Neg	128	31	53	288	138	34	74	258
	Pos	568	159	129	1371	736	301	198	2871
TA1535	Neg	12	5	1	45	12	4	1	42
	Pos	378	164	6	978	104	84	18	1640
TA1537	Neg	6	3	0	30	7	3	1	29
	Pos	708	409	13	2786	88	106	12	2060
WP2 *uvr*A	Neg	14	5	4	48	16	6	4	115
	Pos	190	138	34	961	317	299	22	2632

SD=standard deviation; Min=minimum value; Max=maximum value; Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

APPENDIX B

Study Protocol

QA APPROVED 6DS 12/6/01

Received by RA/OA 06-Dec-2001

Sponsor Project Number: DuPont-8868

BioReliance Study Number: AA52XL502001.BTL

H-25171: Bacterial Reverse Mutation Test with an Independent Repeat Assay

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of S9 activation.

2.0 SPONSOR

2.1	Name:	E.I. du Pont de Nemours and Company
2.2	Address:	Stine Haskell Research Center DuPont Haskell Laboratory P.O. Box 50 1090 Elkton Road Newark, DE 19714-0050
2.3	Representative:	Maria Donner, Ph.D. Phone: 302-366-5251 Fax: 302-366-5207 Email: maria.donner@usa.dupont.com
2.4	Sponsor Project No.:	DuPont-8868
2.5	WR#:	[redacted]
2.6	Haskell #:	H-25171
2.7	Service Code:	[redacted]

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Substance Name:		[redacted]
3.2	Test Substance I.D.:		H-25171 (to be used in the report title and text)
3.3	Controls:	Negative:	Test substance vehicle
		Positive:	9-aminoacridine 2-aminoanthracene methyl methanesulfonate 2-nitrofluorene sodium azide

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3.4 Test Substance Characterization

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 substance, and the stability and strength of the test substance in the solvent (or vehicle).

3.5 Test Substance Retention Sample

The retention of a reserve sample of the test substance 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: Valentine O. Wagner III, M.S.
Phone: 301-610-2152
Fax: 301-738-2362
Email: swagner@bioreliance.com

5.0 PROPOSED STUDY DATES

5.1 Experimental Start Date: 07-Dec-2001

5.2 Experimental Termination Date: 25-Jan-2002

5.3 Draft Report Date: 08-Feb-2002

5.4 Final Report Date: 2 weeks after Sponsor approves draft

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976).

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	rfa	Δ*uvr*B	-

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Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA100	-	TA98	-	rfa	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

Each *S. typhimurium* tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the *uvr*B gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The *E. coli* tester strain has an AT base pair at the critical mutation site within the *trp*E gene (Wilcox *et al.*, 1990). Tester strain WP2 *uvr*A has a deletion in the *uvr*A gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations (Green and Muriel, 1976).

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test substance will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in triplicate.

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7.1 Solubility Determination

Unless the Sponsor has indicated the test substance 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, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). 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.

7.2 Preliminary Toxicity Assay to Select Dose Levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test substance assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA98, TA100, TA1535, TA1537 and WP2 *uvr*A to negative controls and to at least eight concentrations of test substance, one plate per dose level, in both the presence and absence of S9 activation. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. In selecting dose levels for the mutagenicity assay the following guidelines will be employed. Doses will be selected such that precipitate does not interfere with manual scoring. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity without exceeding 5 mg/plate. For freely soluble, nontoxic test substances, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test substances, the highest dose level will be selected in an attempt to yield precipitate at only the top one or two dose levels. The Sponsor will be consulted regarding dose selection if (1) the maximum dose level is selected based on precipitation and this dose level is less than 5 mg/plate or (2) the maximum achievable test substance dose level is less than 5 mg/plate and this dose level is nontoxic.

7.3 Frequency and Route of Administration

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

After the data generated in the first assay have been evaluated, the mutagenicity assay will be repeated. The dose levels used in the second assay will be the same as those used in the first assay unless the Study Director determines that the dose levels should be changed due to an equivocal response, excessive cytotoxicity or

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excessive precipitate. If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay. (e.g., activation system or treatment method). This guidance is based on the OECD Guideline 471 (adopted July 1997 and published February 1998) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1997).

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (μg/plate)
Salmonella Strains	Rat	2-aminoanthracene	1.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene	1.0
TA100, TA1535		sodium azide	1.0
TA1537		9-aminoacridine	75
WP2 *uvr*A		methyl methanesulfonate	1,000

7.4.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone, unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

7.4.3 Sterility Controls

The most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately

-70∘C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2∘C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test substance, test phase, dose level, tester strain and activation type as described in BioReliance's Standard Operating Procedures.

7.8 Test Substance Preparation

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under yellow light.

7.9 Treatment of Test System

One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 μL of tester strain and 50 μL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2∘C. When necessary to achieve the target concentration or eliminate toxic vehicle effects, aliquots of other than 50 μL of test substance/vehicle/positive control will be plated. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar.

After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.10 Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all tester strain cultures will be checked for the appropriate genetic markers cited in §6.0.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

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

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit characteristic number of spontaneous revertants per plate in the negative controls (vehicle). The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

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8.4 Positive Control Values

Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean negative control value (vehicle) for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean negative control value (vehicle).

9.2 Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean negative control value (vehicle).

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. The report will include:

- 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 substance, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.

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- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure [redacted] 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.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Assay), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (GLPs). The protocol, an in-process phase, the raw data, and report(s) will be audited 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. The in-process inspection will be performed to audit the critical assay procedures and systems supporting the assay. A signed QA statement will be included in the final report. This statement will list the system phases inspected during the previous quarter or the study-specific phases, 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 guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test or control substances or their mixtures.

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

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp$^+$ reversion in *Escherichia coli*. Mutation Research 38:3-32.

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.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis 5:285-291.

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14.0 APPROVAL

Marie Donner — 03-DEC-2001
Sponsor Representative — Date

Maria Donner
(Print or Type Name)

Valentine O. Wagner, III — 5 Dec 2001
BioReliance Study Director — Date

Richard — 06 Dec 2001
BioReliance Study Management — Date

APPENDIX C

Information for Japanese Regulatory Agencies

Report of Results of Reverse-Mutation Test in Bacteria

1. Tester Strains

(1) Procurement

Strain	Obtained from	Date obtained	Date inspected the strain lot in storage
TA98	Dr. Bruce Ames University of California, Berkeley	10 November 1998	The genetic markers for each culture are confirmed on the day of use
TA100		11 August 1998	
TA1535			
TA1537			
TA1538			
TA97		13 December 1990	
TA102		14 November 1990	
WP2 *uvr*A	National Collection of Industrial and Marine Bacteria Aberdeen, Scotland	1 July 1987	
WP2 *uvr*A (pkM101)			
WP2 (pKM101)		19 February 1993	

(2) Storage

Freezing method	Large quantity	
Storage temperature	-70°C	
Composition	Bacterial suspension	1.0 mL
	DMSO	0.09 mL

2. S9 Mix

(1) Source, Storage Temperature, etc. of S9

Made in-house	Prepared on	26 November 2001 (Batch R656)	
Storage temperature	-70°C or colder	Name and model of storage apparatus	So-Low, Model PR27-120

(2) Preparation of S9

Animal used		Inducing substance	
Species, Strain	Rattus norvegicus, Sprague Dawley	Name	Aroclor 1254
Sex	Male	Administration method	intraperitoneal
Age (in weeks)	9 (Batch R656)	Administration period and amount (g/kg-weight)	5 days, 0.5 gm/kg body weight
Weight	187 to 271 g (Batch R656)		

3. Preparation of Test Substance Solution

Solvent used			
Name	Manufacturer	Lot No.	Grade and/or Purity (%)
Tetrahydrofuran (CAS No. 109-99-9)	Aldrich Chemical Company	J100352HI	Gold Label, 99.9%
Stability of test substance in the solvent	Unknown		
Method of suspension when test substance is difficult to dissolve	Sonication and warming to 50°C in screw-cap tubes.		

4. Conditions of Pre-culture

Nutrient broth	Name	Manufacturer	Lot No.
	Oxoid Nutrient Broth No. 2	Oxoid Ltd.	CH,-B=210287
Period of pre-culture	12±1 hours		
Storage time and temp. from inoculation to beginning of shaking culture	2 to 5 hours at ambient temperature		
Storage time and temp. from end of culture to use for test	<8 hours at 2-8°C		
Model and manufacturer of shaker	New Brunswick Scientific, model G-24		
Method of shaking (shaking type, speed, etc.)	Rotary (125 rev/min.)		
Culture vessel (shape, capacity)	shape: cylinder, 200 mL		
Culture volume	50 mL		
Volume of inoculum	1 colony		

5. Agar Plate Medium

(1) Top agar

Agar	Name	BBL Select
	Manufacturer	Becton Dickinson
	Lot No.	1000J3DKSQ

(2) Minimum Glucose Agar

Made in-house	Agar	Name	BBL Select
		Manufacturer	Becton Dickinson
		Lot No.	1000J3DKSQ
	Volume of agar plate medium		25 mL

6. Test Results - Judgement of the results

Judgement	Negative
Reason for judgement and referential matters: No positive response was observed with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.	

Referential matters

The vehicle and positive control values indicate that all tester strains were functioning correctly and were capable of detecting a mutagen.