TRADE SECRET

Study Title

[redacted] Bacterial Reverse Mutation Test with an Independent Repeat Assay

Authors

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Study Completion Date

16 September 2002

Performing Laboratory

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Performing Laboratory Study Number

AA60ES.502001.BTL

Work Request Number

[redacted]

Service Code

[redacted]

STATEMENT OF COMPLIANCE

Study N[redacted] 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 stability of the test substance has not been determined by the testing facility.

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

Valentine O. Wagner, III — 16 Sep 2002

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

[redacted] — 16 Sep 2002

BioReliance Study Management — Date

Quality Assurance Statement

Study Title: BACTERIAL REVERSE MUTATION ASSAY WITH AN INDEPENDENT REPEAT ASSAY ASSAY

Study Number: [redacted]

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	14-Jun-02 - 14-Jun-02 To Study Dir 14-Jun-02 To Mgmt 14-Jun-02
	Phase	Protocol Review
**	Inspect On	18-Jun-02 - 18-Jun-02 To Study Dir 18-Jun-02 To Mgmt 20-Jun-02
	Phase	Preparation of S9 mixture
**	Inspect On	20-Aug-02 - 20-Aug-02 To Study Dir 20-Aug-02 To Mgmt 29-Aug-02
	Phase	Draft Report
**	Inspect On	11-Sep-02 - 11-Sep-02 To Study Dir 11-Sep-02 To Mgmt 16-Sep-02
	Phase	Draft to Final Report
*	Inspect On	04-Jun-02 - 04-Jun-02 To Study Dir 04-Jun-02 To Mgmt 05-Jun-02
	Phase	Preparation of S9 mixture
*	Inspect On	12-Jun-02 - 12-Jun-02 To Study Dir 12-Jun-02 To Mgmt 13-Jun-02
	Phase	Dilution of test and/or control material
*	Inspect On	27-Jun-02 - 27-Jun-02 To Study Dir 27-Jun-02 To Mgmt 28-Jun-02
	Phase	Test and/or control material administration

*	Inspect On	02-Jul-02 - 02-Jul-02 To Study Dir 02-Jul-02 To Mgmt 03-Jul-02
	Phase	Test and/or control material administration
*	Inspect On	09-Jul-02 - 09-Jul-02 To Study Dir 09-Jul-02 To Mgmt 09-Jul-02
	Phase	Dilution of test and/or control material
*	Inspect On	17-Jul-02 - 17-Jul-02 To Study Dir 17-Jul-02 To Mgmt 17-Jul-02
	Phase	Strain characterization
*	Inspect On	25-Jul-02 - 25-Jul-02 To Study Dir 25-Jul-02 To Mgmt 30-Jul-02
	Phase	Test and/or control material administration
*	Inspect On	31-Jul-02 - 31-Jul-02 To Study Dir 31-Jul-02 To Mgmt 01-Aug-02
	Phase	Scoring the plates
*	Inspect On	07-Aug-02 - 07-Aug-02 To Study Dir 07-Aug-02 To Mgmt 13-Aug-02
	Phase	Preparation of S9 mixture
*	Inspect On	21-Aug-02 - 21-Aug-02 To Study Dir 21-Aug-02 To Mgmt 28-Aug-02
	Phase	Strain characterization
*	Inspect On	27-Aug-02 - 27-Aug-02 To Study Dir 27-Aug-02 To Mgmt 28-Aug-02
	Phase	Test and/or control material administration

** Inspection specific for this study
* Systems Inspection

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

M Bauernschub 16 Sept 2002

Marcie Bauernschub, B.S. 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: Valentine O. Wagner, III — 16 Sep 2002
Valentine O. Wagner III, M.S. — Date

Approved by Study Monitor: [signature] — 06 SEP 2002
Maria Donner, Ph.D.
Senior Research Toxicologist — Date

TABLE OF CONTENTS

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes:
- [redacted]
- H-25339
- [redacted]

Haskell Number: 25339

Composition: [redacted]

Known Impurities: [redacted]

Physical Characteristics: Yellow dispersion in water

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: June 13, 2002 / (see report cover page)

In-Life Initiated/Completed: June 18, 2002 / July 19, 2002

SUMMARY

The test substance, H-25339, 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.

Water was selected as the solvent of choice based on the Sponsor's request.

In the preliminary toxicity test, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The test substance was soluble but cloudy in water at approximately 50 mg/mL. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Neither precipitate nor 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 100, 333, 1000, 3333 and 5000 µg per plate. Neither precipitate nor 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-25339 did not cause a positive 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.

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).

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, H-25339, was received by BioReliance on 12 June 2002 and was assigned the code number [redacted] The test substance was characterized by the Sponsor as yellow dispersion in water that should be stored at ambient temperature. An expiration date of 01 October 2002 was provided. Upon receipt, the test substance was described as a pale yellow cloudy liquid and was stored at room temperature and in the dark.

The identity, strength, purity, composition or other characteristics to define the test substance have been determined by the Sponsor. The stability of the test substance has been determined by the Sponsor but a copy of the analysis was not provided to BioReliance.

The vehicle used to deliver H-25339 to the test system was sterile distilled water (CAS No. 7732-18-5), obtained from Invitrogen Corporation. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light.

The negative and positive control substances have been characterized as per the Certificates on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that meet the criteria for a valid test.

Positive controls plated concurrently with the mutagenicity test are listed in the following table. 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 97.4%	1.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene (Aldrich Chemical Co., Inc.) Lot No. 08707HS Exp. Date 08-Mar-2006 CAS No. 607-57-8 Purity 99.9%	1.0
TA100, TA1535		Sodium azide (Sigma Chemical Co.) Lot No. 098H0169 Exp. Date 05-Jan-2004 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Chemical Co.) Lot No. 106F06681 Exp. Date 18-Nov-2004 CAS No. 90-45-9 Purity >97%	75
WP2 *uvr*A		Methyl methanesulfonate (Aldrich Chemical Co., Inc.) Lot No. 15526AO Exp. Date 28-Nov-2003 CAS No. 66-27-3 Purity 99.9%	1,000

To confirm the sterility of the test substance, the highest test substance dose level used in the mutagenicity test 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. *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.

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 by BioReliance or purchased from Moltox 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

Water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

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. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate.

Mutagenicity Test

The mutagenicity test was used to evaluate the mutagenic potential of the test substance. Five dose levels of test substance 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. The dose levels tested were 100, 333, 1000, 3333 and 5000 µg per plate. 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 100 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 in the following table.

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.
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).

Code	Description	Characteristics
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 $0.3x10^9$ 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) At least a moderate reduction in the background lawn (background lawn code 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 test-method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Water was selected as the solvent of choice based on the Sponsor's request.

Preliminary Toxicity Test

The results of the preliminary toxicity test are presented in Tables 1 through 5. 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 50 mg/mL and a 100 µL plating aliquot. The test substance was soluble but cloudy in water at approximately 50 mg/mL. The dose levels tested were 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg per plate. Neither precipitate nor 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.

Mutagenicity Test

The results of the mutagenicity test are presented in Tables 6 through 25 and summarized in Tables 26 and 27. These data were generated in Experiments B1 and B2. The dose levels tested were 100, 333, 1000, 3333 and 5000 µg per plate. Neither precipitate nor appreciable toxicity was observed.

In Experiment B1 (Initial 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 Assay), 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-25339 did not cause a positive 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.

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.

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-25339
Study Number : [redacted]
Experiment No. : A1
Strain : TA98
Date Plated : 18 Jun 2002
Vehicle : water
Plating Aliquot : 100 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	11	1	13	1
6.7	17	1	13	1
10	17	1	10	1
33	15	1	13	1
67	17	1	10	1
100	18	1	12	1
333	17	1	14	1
667	20	1	14	1
1000	19	1	11	1
3333	17	1	11	1
5000	19	1	13	1

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-25339
Study Number : [redacted]
Experiment No. : A1
Strain : TA100
Date Plated : 18 Jun 2002
Vehicle : water
Plating Aliquot : 100 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	117	1	171	1
6.7	133	1	169	1
10	146	1	175	1
33	153	1	175	1
67	160	1	200	1
100	152	1	181	1
333	194	1	216	1
667	181	1	160	1
1000	182	1	178	1
3333	192	1	198	1
5000	181	1	212	1

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 3

Test Substance Id: H-25339
Study Number : [redacted]
Experiment No. : A1
Strain : TA1535
Date Plated : 18 Jun 2002
Vehicle : water
Plating Aliquot : 100 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	16	1	7	1
6.7	11	1	9	1
10	11	1	10	1
33	8	1	6	1
67	10	1	11	1
100	12	1	10	1
333	12	1	10	1
667	12	1	10	1
1000	11	1	12	1
3333	13	1	10	1
5000	11	1	12	1

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-25339
Study Number : [redacted]
Experiment No. : A1
Strain : TA1537
Date Plated : 18 Jun 2002
Vehicle : water
Plating Aliquot : 100 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	10	1	10	1
6.7	13	1	8	1
10	13	1	9	1
33	11	1	11	1
67	8	1	8	1
100	7	1	6	1
333	6	1	8	1
667	7	1	6	1
1000	6	1	7	1
3333	8	1	8	1
5000	5	1	5	1

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 5

Test Substance Id: H-25339
Study Number : [redacted]
Experiment No. : A1
Strain : WP2 uvrA
Date Plated : 18 Jun 2002
Vehicle : water
Plating Aliquot : 100 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	12	1	24	1
6.7	12	1	17	1
10	13	1	16	1
33	15	1	16	1
67	13	1	16	1
100	13	1	16	1
333	14	1	15	1
667	14	1	10	1
1000	18	1	13	1
3333	16	1	14	1
5000	16	1	16	1

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-25339
Study Number : [redacted]
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.8 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	15	1		
	02	15	1		
	03	14	1	15	1
100	01	16	1		
	02	13	1		
	03	17	1	15	2
333	01	12	1		
	02	14	1		
	03	17	1	14	3
1000	01	12	1		
	02	14	1		
	03	12	1	13	1
3333	01	14	1		
	02	13	1		
	03	12	1	13	1
5000	01	13	1		
	02	14	1		
	03	15	1	14	1
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	118	1		
	02	144	1		
	03	101	1	121	22

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-25339
Study Number : [redacted]
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.8 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	13	1		
	03	16	1	14	2
100	01	14	1		
	02	13	1		
	03	12	1	13	1
333	01	12	1		
	02	12	1		
	03	12	1	12	0
1000	01	14	1		
	02	13	1		
	03	12	1	13	1
3333	01	13	1		
	02	12	1		
	03	14	1	13	1
5000	01	13	1		
	02	14	1		
	03	14	1	14	1
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	398	1		
	02	312	1		
	03	381	1	364	46

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 8

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA100
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 1.5 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	150	1		
	02	175	1		
	03	163	1	163	13
100	01	160	1		
	02	181	1		
	03	174	1	172	11
333	01	209	1		
	02	169	1		
	03	175	1	184	22
1000	01	154	1		
	02	193	1		
	03	155	1	167	22
3333	01	216	1		
	02	229	1		
	03	228	1	224	7
5000	01	266	1		
	02	247	1		
	03	247	1	253	11
Positive Control sodium azide 1.0 µg per plate					
	01	665	1		
	02	693	1		
	03	696	1	685	17

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-25339
Study Number : [redacted] Experiment No : B1
Strain : TA100 Cells Seeded : 1.5 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 2 Jul 2002
Vehicle : water
Plating Aliquot : 100 µL

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	191	1		
	02	174	1		
	03	177	1	181	9
100	01	190	1		
	02	159	1		
	03	185	1	178	17
333	01	168	1		
	02	141	1		
	03	184	1	164	22
1000	01	92	1		
	02	135	1		
	03	165	1	131	37
3333	01	210	1		
	02	194	1		
	03	150	1	185	31
5000	01	197	1		
	02	199	1		
	03	181	1	192	10
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	890	1		
	02	747	1		
	03	703	1	780	98

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-25339
Study Number : [redacted]
Strain : TA1535
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.2 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	15	1		
	02	10	1		
	03	13	1	13	3
100	01	13	1		
	02	12	1		
	03	12	1	12	1
333	01	13	1		
	02	12	1		
	03	15	1	13	2
1000	01	12	1		
	02	11	1		
	03	12	1	12	1
3333	01	10	1		
	02	10	1		
	03	10	1	10	0
5000	01	12	1		
	02	10	1		
	03	12	1	11	1
Positive Control sodium azide 1.0 µg per plate					
	01	425	1		
	02	370	1		
	03	355	1	383	37

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-25339
Study Number : [redacted]
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.2 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	10	1		
	03	11	1	11	1
100	01	11	1		
	02	10	1		
	03	12	1	11	1
333	01	11	1		
	02	13	1		
	03	12	1	12	1
1000	01	10	1		
	02	10	1		
	03	10	1	10	0
3333	01	14	1		
	02	10	1		
	03	10	1	11	2
5000	01	11	1		
	02	10	1		
	03	10	1	10	1
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	91	1		
	02	95	1		
	03	87	1	91	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 12

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA1537
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 1.2 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	6	1		
	02	7	1		
	03	12	1	8	3
100	01	4	1		
	02	9	1		
	03	12	1	8	4
333	01	12	1		
	02	7	1		
	03	11	1	10	3
1000	01	10	1		
	02	6	1		
	03	13	1	10	4
3333	01	10	1		
	02	10	1		
	03	11	1	10	1
5000	01	10	1		
	02	10	1		
	03	14	1	11	2
Positive Control 9-aminoacridine 75 µg per plate					
	01	632	1		
	02	698	1		
	03	527	1	619	86

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-25339
Study Number : [redacted]
Strain : TA1537
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 1.2 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	4	1		
	02	4	1		
	03	7	1	5	2
100	01	5	1		
	02	7	1		
	03	5	1	6	1
333	01	7	1		
	02	6	1		
	03	6	1	6	1
1000	01	7	1		
	02	7	1		
	03	9	1	8	1
3333	01	7	1		
	02	7	1		
	03	8	1	7	1
5000	01	8	1		
	02	10	1		
	03	4	1	7	3
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	54	1		
	02	52	1		
	03	50	1	52	2

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-25339
Study Number : [redacted]
Strain : WP2 uvrA
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 3.7×10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	10	1		
	02	10	1		
	03	13	1	11	2
100	01	13	1		
	02	15	1		
	03	14	1	14	1
333	01	11	1		
	02	14	1		
	03	13	1	13	2
1000	01	13	1		
	02	13	1		
	03	14	1	13	1
3333	01	14	1		
	02	16	1		
	03	12	1	14	2
5000	01	14	1		
	02	14	1		
	03	16	1	15	1
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	103	1		
	02	96	1		
	03	86	1	95	9

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-25339
Study Number : [redacted]
Strain : WP2 uvrA
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 3.7 X 10^8
Date Plated : 2 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	C			
	02	14	1		
	03	15	1	15	1
100	01	12	1		
	02	12	1		
	03	11	1	12	1
333	01	14	1		
	02	14	1		
	03	12	1	13	1
1000	01	16	1		
	02	14	1		
	03	14	1	15	1
3333	01	12	1		
	02	16	1		
	03	15	1	14	2
5000	01	11	1		
	02	15	1		
	03	15	1	14	2
Positive Control 2-aminoanthracene 10 µg per plate					
	01	548	1		
	02	552	1		
	03	471	1	524	46

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 16

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 2.4 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	16	1		
	02	14	1		
	03	12	1	14	2
100	01	16	1		
	02	19	1		
	03	16	1	17	2
333	01	19	1		
	02	22	1		
	03	20	1	20	2
1000	01	23	1		
	02	19	1		
	03	23	1	22	2
3333	01	18	1		
	02	17	1		
	03	20	1	18	2
5000	01	14	1		
	02	17	1		
	03	16	1	16	2
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	287	1		
	02	225	1		
	03	303	1	272	41

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-25339
Study Number : [redacted]
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 2.4 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	17	1		
	02	15	1		
	03	16	1	16	1
100	01	13	1		
	02	19	1		
	03	12	1	15	4
333	01	20	1		
	02	17	1		
	03	19	1	19	2
1000	01	19	1		
	02	20	1		
	03	17	1	19	2
3333	01	18	1		
	02	13	1		
	03	12	1	14	3
5000	01	19	1		
	02	16	1		
	03	15	1	17	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	151	1		
	02	132	1		
	03	134	1	139	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 18

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA100
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.4 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	201	1		
	02	215	1		
	03	167	1	194	25
100	01	178	1		
	02	198	1		
	03	185	1	187	10
333	01	187	1		
	02	213	1		
	03	241	1	214	27
1000	01	C			
	02	192	1		
	03	192	1	192	0
3333	01	208	1		
	02	210	1		
	03	201	1	206	5
5000	01	201	1		
	02	215	1		
	03	217	1	211	9
Positive Control sodium azide 1.0 µg per plate					
	01	574	1		
	02	671	1		
	03	563	1	603	59

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 19

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA100
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.4 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	164	1		
	02	118	1		
	03	141	1	141	23
100	01	194	1		
	02	176	1		
	03	175	1	182	11
333	01	168	1		
	02	167	1		
	03	143	1	159	14
1000	01	148	1		
	02	197	1		
	03	188	1	178	26
3333	01	161	1		
	02	148	1		
	03	156	1	155	7
5000	01	159	1		
	02	154	1		
	03	163	1	159	5
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	758	1		
	02	781	1		
	03	748	1	762	17

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-25339
Study Number : [redacted]
Strain : TA1535
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.9 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	18	1		
	03	18	1	16	3
100	01	11	1		
	02	14	1		
	03	10	1	12	2
333	01	12	1		
	02	12	1		
	03	13	1	12	1
1000	01	12	1		
	02	18	1		
	03	9	1	13	5
3333	01	14	1		
	02	13	1		
	03	12	1	13	1
5000	01	12	1		
	02	12	1		
	03	13	1	12	1
Positive Control sodium azide 1.0 µg per plate					
	01	342	1		
	02	368	1		
	03	368	1	359	15

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 21

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.9 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	8	1		
	03	8	1	9	2
100	01	10	1		
	02	8	1		
	03	11	1	10	2
333	01	8	1		
	02	12	1		
	03	11	1	10	2
1000	01	11	1		
	02	13	1		
	03	6	1	10	4
3333	01	6	1		
	02	9	1		
	03	9	1	8	2
5000	01	10	1		
	02	9	1		
	03	12	1	10	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	114	1		
	02	121	1		
	03	120	1	118	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 22

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA1537
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.1 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	5	1		
	02	8	1		
	03	4	1	6	2
100	01	6	1		
	02	10	1		
	03	3	1	6	4
333	01	10	1		
	02	5	1		
	03	4	1	6	3
1000	01	8	1		
	02	10	1		
	03	6	1	8	2
3333	01	5	1		
	02	7	1		
	03	9	1	7	2
5000	01	9	1		
	02	8	1		
	03	3	1	7	3
Positive Control 9-aminoacridine 75 µg per plate					
	01	369	1		
	02	356	1		
	03	404	1	376	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 23

Test Substance Id: H-25339
Study Number : [redacted]
Strain : TA1537
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 1.1 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	3	1		
	02	4	1		
	03	4	1	4	1
100	01	4	1		
	02	7	1		
	03	3	1	5	2
333	01	7	1		
	02	6	1		
	03	7	1	7	1
1000	01	7	1		
	02	6	1		
	03	7	1	7	1
3333	01	3	1		
	02	3	1		
	03	6	1	4	2
5000	01	4	1		
	02	6	1		
	03	3	1	4	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	110	1		
	02	114	1		
	03	113	1	112	2

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 24

Test Substance Id: H-25339
Study Number : [redacted]
Strain : WP2 uvrA
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B2
Cells Seeded : 4.0 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	16	1		
	02	16	1		
	03	10	1	14	3
100	01	9	1		
	02	9	1		
	03	16	1	11	4
333	01	14	1		
	02	13	1		
	03	14	1	14	1
1000	01	14	1		
	02	18	1		
	03	12	1	15	3
3333	01	8	1		
	02	11	1		
	03	10	1	10	2
5000	01	18	1		
	02	15	1		
	03	17	1	17	2
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	106	1		
	02	117	1		
	03	83	1	102	17

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 25

Test Substance Id: H-25339
Study Number : [redacted]
Strain : WP2 uvrA
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL
Experiment No : B2
Cells Seeded : 4.0 X 10^8
Date Plated : 11 Jul 2002

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	11	1		
	03	13	1	13	2
100	01	15	1		
	02	13	1		
	03	18	1	15	3
333	01	15	1		
	02	12	1		
	03	14	1	14	2
1000	01	11	1		
	02	15	1		
	03	14	1	13	2
3333	01	13	1		
	02	12	1		
	03	12	1	12	1
5000	01	14	1		
	02	10	1		
	03	17	1	14	4
Positive Control 2-aminoanthracene 10 µg per plate					
	01	122	1		
	02	115	1		
	03	131	1	123	8

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 26

Test Substance Id : H-25339
Study Number : [redacted] Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	15 ± 1	163 ± 13	13 ± 3	8 ± 3	11 ± 2
100	15 ± 2	172 ± 11	12 ± 1	8 ± 4	14 ± 1
333	14 ± 3	184 ± 22	13 ± 2	10 ± 3	13 ± 2
1000	13 ± 1	167 ± 22	12 ± 1	10 ± 4	13 ± 1
3333	13 ± 1	224 ± 7	10 ± 0	10 ± 1	14 ± 2
5000	14 ± 1	253 ± 11	11 ± 1	11 ± 2	15 ± 1
Positive	121 ± 22	685 ± 17	383 ± 37	619 ± 86	95 ± 9

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	14 ± 2	181 ± 9	11 ± 1	5 ± 2	15 ± 1
100	13 ± 1	178 ± 17	11 ± 1	6 ± 1	12 ± 1
333	12 ± 0	164 ± 22	12 ± 1	6 ± 1	13 ± 1
1000	13 ± 1	131 ± 37	10 ± 0	8 ± 1	15 ± 1
3333	13 ± 1	185 ± 31	11 ± 2	7 ± 1	14 ± 2
5000	14 ± 1	192 ± 10	10 ± 1	7 ± 3	14 ± 2
Positive	364 ± 46	780 ± 98	91 ± 4	52 ± 2	524 ± 46

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 100 µL

Bacterial Mutation Test
Summary of Results

Table 27

Test Substance Id : H-25339
Study Number [redacted] Experiment No : B2

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	14 ± 2	194 ± 25	16 ± 3	6 ± 2	14 ± 3
100	17 ± 2	187 ± 10	12 ± 2	6 ± 4	11 ± 4
333	20 ± 2	214 ± 27	12 ± 1	6 ± 3	14 ± 1
1000	22 ± 2	192 ± 0	13 ± 5	8 ± 2	15 ± 3
3333	18 ± 2	206 ± 5	13 ± 1	7 ± 2	10 ± 2
5000	16 ± 2	211 ± 9	12 ± 1	7 ± 3	17 ± 2
Positive	272 ± 41	603 ± 59	359 ± 15	376 ± 25	102 ± 17

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	16 ± 1	141 ± 23	9 ± 2	4 ± 1	13 ± 2
100	15 ± 4	182 ± 11	10 ± 2	5 ± 2	15 ± 3
333	19 ± 2	159 ± 14	10 ± 2	7 ± 1	14 ± 2
1000	19 ± 2	178 ± 26	10 ± 4	7 ± 1	13 ± 2
3333	14 ± 3	155 ± 7	8 ± 2	4 ± 2	12 ± 1
5000	17 ± 2	159 ± 5	10 ± 2	4 ± 2	14 ± 4
Positive	139 ± 10	762 ± 17	118 ± 4	112 ± 2	123 ± 8

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 100 µL

APPENDIX A

Historical Control Data

Historical Negative and Positive Control Values
1999 – 2001

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	16	6	4	59	21	7	6	58
	Pos	310	191	21	1581	680	374	40	2294
TA100	Neg	143	37	53	283	149	39	74	271
	Pos	564	160	129	1851	876	438	163	2922
TA1535	Neg	14	6	1	46	13	5	1	43
	Pos	335	149	6	978	125	84	11	1640
TA1537	Neg	6	3	0	30	7	3	1	29
	Pos	732	391	15	2786	117	130	12	2060
WP2 *uvr*A	Neg	14	4	5	52	15	5	4	115
	Pos	168	140	34	990	322	286	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

Received by RA/QA 13-Jun-2002

Sponsor Project Number: DuPont-10819

BioReliance Study Number: [redacted]

[redacted] Bacterial Reverse Mutation Test
with an Independent Repeat Assay

QA 6/14/02
APPROVED

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:	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-10819
2.5	WR#:	[redacted]
2.6	Haskell #:	H-25339
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-25339 (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

Sponsor Project Number: DuPont-10819

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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: 02-Jul-2002

5.2 Experimental Termination Date: 20-Aug-2002

5.3 Draft Report Date: 03-Sep-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

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

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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 *uvrA*

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.

- 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 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.

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

Maria Donner — 10 JUN 2002
Sponsor Representative — Date

Maria Donner
(Print or Type Name)

Valentine O. Wagner, III — 13 Jun 2002
BioReliance Study Director — Date

[signature] — 13 Jun 2002
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	13 February 2002 (Batch R663)	
Purchased from Moltox	Prepared on	18 April 2002 (Lot 1384) 14 May 2002 (Lot 1390)	
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 R663) unknown (Lot 1384) unknown (Lot 1390)	Administration period and amount (g/kg-weight)	single dose at 0.5 gm/kg body weight, sacrificed after 5 days
Weight	211 to 251 g (Batch R663) unknown (Lot 1384) unknown (Lot 1390)		

3. Preparation of Test Substance Solution

Solvent used			
Name	Manufacturer	Lot No.	Grade and/or Purity (%)
Water (CAS No. 7732-18-5)	Invitrogen Corporation	1129947	Distilled
Stability of test substance in the solvent	Unknown		
Method of suspension when test substance is difficult to dissolve	Not applicable		

4. Conditions of Pre-culture

Nutrient broth	Name	Manufacturer	Lot No.
	Oxoid Nutrient Broth No. 2	Oxoid Ltd.	CH,-B=246975
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 either the presence or 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.