Final Report

TRADE SECRET

Study Title

H-22051: MUTAGENICITY TESTING
IN THE *SALMONELLA TYPHIMURIUM* AND
ESCHERICHIA COLI PLATE INCORPORATION ASSAY

Laboratory Project ID

Haskell Laboratory Report No. 1997-00035

Authors

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Study Completed on

04-02-97

Performing Laboratory

E. I. du Pont de Nemours and Company
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Newark, Delaware 19714

Microbiological Associates, Inc. Study No. G96CF43.502011

Medical Research Project No. [redacted]

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with EPA FIFRA (40 CFR 160) EPA TSCA (40 CFR 792) Good Laboratory Practice Standards, OECD Principles of Good Laboratory Practice (C(81)30(Final), Annex 2), and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850) with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Treatment solutions or suspensions were not analyzed for identity, composition, uniformity, or stability of the test and control articles. The procedures used by trained personnel to prepare the treatment solutions are intended to ensure:

A. The accuracy of concentration because the test and control articles were weighed on an analytical balance accurate to 3 decimal places and the vehicle in which the test and control substances were dissolved was accurately measured with graduated pipets or flasks;

B. Uniformity because all treatment solutions or suspensions were mixed prior to administration to the test system; and

C. Stability because treatment solutions or suspensions were prepared just prior to administration to the test system.

These deviations did not affect the validity or the integrity of the study.

Submitter: E. I. du Pont de Nemours and Company

Sponsor: DuPont Specialty Chemicals

Study Director: Valentine O. Wagner, III — 2 April 1997
Valentine O. Wagner, III, M.S. — Date
Microbiological Associates, Inc.

Study Monitor: [signature] — 4/10/97
Brian H. Mathison, Ph.D. — Date
Research Toxicologist

GENERAL INFORMATION

Test Substance: [redacted]

Codes/Synonyms: • H-2205! [redacted]

Haskell Number: 22051

CAS Registry Number: [redacted]

Stability: Test substance appeared to be stable under the condition of the study; no evidence of instability was observed.

Composition: [redacted]

Physical State: Liquid

Major Impurities: Unknown, none considered to be of toxicological significance at this time.

Sponsor: DuPont Specialty Chemicals
E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898

Study Initiated/Completed: November 27, 1996 / April 2, 1997

In-Life Initiated/Completed: December 3, 1996 / January 6, 1997

Archives

All raw data, and reports will be maintained in the archives of Microbiological Associates, Inc., located in Rockville, Maryland. A sample of the test substance will be archived at E. I. du Pont de Nemours and Company, Haskell Laboratory for Toxicology and Industrial Medicine, Newark, Delaware.

QUALITY ASSURANCE STATEMENT

Study Title: H-22051: MUTAGENICITY TESTING IN THE *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI* PLATE INCORPORATION ASSAY

Study Number: G96CF43.502011

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 Compliance Programme, 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 02 DEC 96, TO STUDY DIR 02 DEC 96, TO MGMT 02 DEC 96
PHASE: Protocol Review

INSPECT ON 03 DEC 96, TO STUDY DIR 06 DEC 96, TO MGMT 06 DEC 96
PHASE: Strain characterization

INSPECT ON 12 DEC 96, TO STUDY DIR 12 DEC 96, TO MGMT 13 DEC 96
PHASE: Preparation of S9 mixture

INSPECT ON 13 FEB 97, TO STUDY DIR 13 FEB 97, TO MGMT 20 FEB 97
PHASE: Draft Report

INSPECT ON 04 APR 97, TO STUDY DIR 04 APR 97, TO MGMT 04 APR 97
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.

Claire L Courtemanche — April 4, 1997

Claire L. Courtemanche, B.S.
QUALITY ASSURANCE — DATE

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SUMMARY

The test article, H-22051, was tested in the bacterial reverse mutation assay using *S. typhimurium* tester strains TA98, TA100, TA1535 and TA97a and *E. coli* tester strain WP2 *uvr*A (pKM101) in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases, using the plate incorporation method. The first phase, the preliminary toxicity assay, was used to establish the dose range for the mutagenicity assay. The second phase, the mutagenicity assay (initial and independent repeat assays), was used to evaluate the mutagenic potential of the test article.

Water was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in water at a concentration of approximately 500 mg/ml, the maximum concentration tested.

In the preliminary toxicity assay, the maximum dose tested was 5000 μg per plate; this dose was achieved using a concentration of 100 mg/ml and a 50 μl plating aliquot. Neither precipitate nor appreciable toxicity was observed. Based on the findings of the toxicity assay, the maximum dose plated in the mutagenicity assay was 5000 μg per plate.

In the mutagenicity assay, no positive response was observed. Neither precipitate nor appreciable toxicity was observed. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation* and Dose Range Tested (μg/plate)									
	TA98		TA100		TA1535		TA97a		WP2 *uvr*A (pKM101)	
	Low	High	Low	High	Low	High	Low	High	Low	High
None	-		-		-		-		-	
	100	5000	100	5000	100	5000	100	5000	100	5000
Rat	-		-		-		-		-	
	100	5000	100	5000	100	5000	100	5000	100	5000

*- = negative, + = positive (maximum fold increase)

Under the conditions of this study, test article H-22051 was concluded to be negative in the Bacterial Reverse Mutation Assay with an Independent Repeat Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and one strain of *E. coli* in the presence and absence of S9 activation.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, H-22051, was received by Microbiological Associates, Inc. on 11/22/96 and was assigned the code number 96CF43. The test article was characterized by the Sponsor as a yellow liquid that should be stored at <30°C. An expiration date was not provided. Upon receipt, the test article was described as a yellow liquid and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver H-22051 to the test system was sterile distilled water, (CAS# 7732-18-5), obtained from Life Technologies, Inc.

Positive controls plated concurrently with the mutagenicity assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA100, TA1535	+	2-aminoanthracene (Sigma Chemical Co.)	1.0
TA97a			2.0
WP2 *uvr*A (pKM101)			10
TA98	-	2-nitrofluorene (Aldrich Chemical Co., Inc.)	1.0
TA100, TA1535		sodium azide (Sigma Chemical Co.)	1.0
TA97a		9-aminoacridine (Sigma Chemical Co.)	75
WP2 *uvr*A (pKM101)		methyl methanesulfonate (Aldrich Chemical Co., Inc.)	1,000

To determine the sterility of the test article, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA97a as described by Ames *et al.* (1975) and Levin *et al.* (1982) and the *E. coli* tester strain WP2 *uvr*A (pKM101) as described by Green and Muriel (1976). *Salmonella* tester strains were received on 11/26/90 and 11/10/92 directly from Dr. Bruce Ames, University of California, Berkeley. *E. coli* was received on 07/01/87 from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Tester strains TA98 and TA97a 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. *E. coli* is sensitive to base-pair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976; Brusick *et al.*, 1980).

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 assays on nutrient agar plates.

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 on 08/07/96, 09/25/96 and 11/25/96 and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed 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 β-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 one or more of the following solvents in the order of preference as listed: purified water, dimethylsulfoxide, ethanol and acetone. The test article was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the dose-range over which the test article would be assayed. Ten dose levels of the test article were plated, one plate per dose, with overnight cultures of TA98, TA100, TA1535, TA97a and WP2 *uvr*A (pKM101) on selective minimal agar in both the presence and absence of rat liver S9 activation.

Mutagenicity Assay

The mutagenicity assay (initial and independent repeat assays) was used to evaluate the mutagenic potential of the test article. A minimum of five dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA97a and WP2 *uvr*A (pKM101) in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in triplicate.

Plating and Scoring Procedures

The test system was exposed to the test article 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 article, test phase, dose level, tester strain, and activation, as described in detail in Microbiological Associates, Inc.'s Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half (0.5) milliliter of S9 or Sham mix, 100 µl of tester strain and 50 µl of vehicle or test article 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 article aliquot was replaced by a 50 or 100 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 4±2°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article 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	Severely 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 ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article 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., ≤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., >3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand unless the assay was the preliminary toxicity assay or the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

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 article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA1535 was judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value. Data sets for strains TA98, TA100, TA97a and WP2 *uvr*A (pKM101) were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity assay 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, TA100, TA97a and WP2 *uvr*A (pKM101) must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A (pKM101) 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; TA97a, 80 - 240; WP2 *uvr*A (pKM101), 150 - 380. 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 three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay 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 reduction in the background lawn.

RESULTS AND DISCUSSION

Solubility Test

Water was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in water at a concentration of approximately 500 mg/ml, the maximum concentration tested.

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in Tables 1 through 5. In the preliminary toxicity assay, the maximum dose tested was 5000 μg per plate; this dose was achieved using a concentration of 100 mg/ml and a 50 μl plating aliquot. Neither precipitate nor appreciable toxicity was observed. Based on the findings of the toxicity assay, the maximum dose plated in the mutagenicity assay was 5000 μg per plate.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 6 through 25 and summarized in Tables 26 and 27. These data were generated in Experiments B1 and B2. Neither precipitate nor appreciable toxicity was observed.

In Experiment B1, the initial mutagenicity assay, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation.

In Experiment B2, the independent repeat assay, no positive responses were observed with any of the tester strains in the presence and 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 Assay with an Independent Repeat Assay indicate that, under the conditions of this study, **H-22051** did not cause a positive response with any of the tester strains in the presence and 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.

Brusick, D.J., Simmon, V.F., Rosenkranz, H.S., Ray, W.A., and R.S. Stafford (1980) An Evaluation of the *Escherichia coli* WP2 and WPA *uvrA* Reverse Mutation Assay. Mutation Research, 76:169-190.

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

Levin, D.E., Yamasaki, E. and B.N. Ames (1982) A new *Salmonella* tester strain, TA97, for the detection of frameshift mutagens. A run of cytosines as a mutational hot-spot, Mutation Research, 94:315-330.

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

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

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 1

Test Article Id : H-22051
Study Number : G96CF43.502011
Experiment No. : A1
Date Plated : 12/03/96
Counted by : hand
Vehicle : water
Plating Aliquot : 50 µl

	TA98			
	With S9 Activation		Without Activation	
Test Article Concentration µg per plate	Revertants per plate	Background Code[a]	Revertants per plate	Background Code[a]
Vehicle	22	1	11	1
6.7	16	1	10	1
10	16	1	15	1
33	19	1	9	1
67	14	1	15	1
100	12	1	8	1
333	23	1	10	1
667	20	1	10	1
1000	16	1	13	1
3333	8	1	12	1
5000	17	1	14	1

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 2

Test Article Id : H-22051
Study Number : G96CF43.502011
Experiment No. : A1
Date Plated : 12/03/96
Counted by : machine
Vehicle : water
Plating Aliquot : 50 µl

	TA100			
	With S9 Activation		Without Activation	
Test Article Concentration µg per plate	Revertants per plate	Background Code[a]	Revertants per plate	Background Code[a]
Vehicle	167	1	165	1
6.7	162	1	143	1
10	198	1	149	1
33	162	1	167	1
67	185	1	147	1
100	191	1	163	1
333	169	1	156	1
667	154	1	165	1
1000	172	1	155	1
3333	162	1	160	1
5000	134	1	159	1

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 3

Test Article Id : H-22051
Study Number : G96CF43.502011
Experiment No. : A1
Date Plated : 12/03/96
Counted by : hand
Vehicle : water
Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA1535 With S9 Activation Revertants per plate	TA1535 With S9 Activation Background Code[a]	TA1535 Without Activation Revertants per plate	TA1535 Without Activation Background Code[a]
Vehicle	14	1	13	1
6.7	12	1	11	1
10	11	1	7	1
33	10	1	12	1
67	8	1	7	1
100	8	1	7	1
333	8	1	8	1
667	7	1	4	1
1000	10	1	11	1
3333	10	1	10	1
5000	8	1	9	1

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 4

Test Article Id : H-22051
Study Number : G96CF43.502011
Experiment No. : A1
Date Plated : 12/03/96
Counted by : machine
Vehicle : water
Plating Aliquot : 50 µl

	TA97a			
	With S9 Activation		Without Activation	
Test Article Concentration µg per plate	Revertants per plate	Background Code[a]	Revertants per pla..	Background Code[a]
Vehicle	179	1	130	1
6.7	137	1	131	1
10	150	1	109	1
33	148	1	121	1
67	151	1	131	1
100	168	1	104	1
333	152	1	127	1
667	172	1	104	1
1000	149	1	124	1
3333	160	1	132	1
5000	121	1	110	1

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 5

Test Article Id : H-22051
Study Number : G96CF43.502011
Experiment No. : A1
Date Plated : 12/03/96
Counted by : machine
Vehicle : water
Plating Aliquot : 50 µl

Test Article Concentration µg per plate	WP2 uvrA (pKM101) With S9 Activation Revertants per plate	With S9 Activation Background Code[a]	Without Activation Revertants per plate	Without Activation Background Code[a]
Vehicle	160	1	135	1
6.7	140	1	152	1
10	153	1	152	1
33	148	1	170	1
67	155	1	135	1
100	113	1	117	1
333	168	1	171	1
667	153	1	145	1
1000	173	1	160	1
3333	136	1	170	1
5000	144	1	140	1

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 6

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B1
Cells Seeded : 1.0 X 10^8
Date Plated : 12/12/96
Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	27	1		
	02	29	1		
	03	18	1	25	6
100	01	26	1		
	02	32	1		
	03	46	1	35	10
333	01	16	1		
	02	20	1		
	03	12	1	16	4
1000	01	25	1		
	02	22	1		
	03	15	1	21	5
3333	01	20	1		
	02	12	1		
	03	20	1	17	5
5000	01	20	1		
	02	20	1		
	03	19	1	20	1
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	100	1		
	02	123	1		
	03	172	1	132	37

[a]Background bacterial evaluation 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

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 7

Test Article Id	: H-22051		
Study Number	: G96CF43.502011	Experiment No	: B1
Strain	: TA98	Cells Seeded	: 1.0 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 12/12/96
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	25	1		
	02	17	1		
	03	15	1	19	5
100	01	28	1		
	02	24	1		
	03	27	1	26	2
333	01	26	1		
	02	27	1		
	03	29	1	27	2
1000	01	33	1		
	02	29	1		
	03	35	1	32	3
3333	01	33	1		
	02	25	1		
	03	39	1	32	7
5000	01	24	1		
	02	31	1		
	03	25	1	27	4
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	719	1		
	02	702	1		
	03	782	1	734	42

[a]Background bacterial evaluation 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

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 8

Test Article Id	: H-22051		
Study Number	: G96CF43.502011	Experiment No	: B1
Strain	: TA100	Cells Seeded	: 0.8 X 10^8
Liver Microsomes	: None	Date Plated	: 12/12/96
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	144	1		
	02	159	1		
	03	131	1	145	14
100	01	116	1		
	02	156	1		
	03	127	1	133	21
333	01	128	1		
	02	150	1		
	03	124	1	134	14
1000	01	147	1		
	02	154	1		
	03	126	1	142	15
3333	01	121	1		
	02	128	1		
	03	138	1	129	9
5000	01	114	1		
	02	135	1		
	03	118	1	122	11
Positive Control sodium azide 1.0 µg per plate					
	01	736	1		
	02	762	1		
	03	743	1	747	13

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 9

Test Article Id	: H-22051		
Study Number	: G96CF43.502011	Experiment No	: B1
Strain	: TA100	Cells Seeded	: 0.8 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 12/12/96
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	153	1		
	02	124	1		
	03	148	1	142	16
100	01	167	1		
	02	129	1		
	03	142	1	146	19
333	01	160	1		
	02	141	1		
	03	154	1	152	10
1000	01	148	1		
	02	166	1		
	03	141	1	152	13
3333	01	136	1		
	02	122	1		
	03	150	1	136	14
5000	01	148	1		
	02	161	1		
	03	147	1	152	8
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	761	1		
	02	778	1		
	03	806	1	782	23

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 10

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA1535
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl
Experiment No : B1
Cells Seeded : 3.0×10^8
Date Plated : 12/12/96
Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	8	1		
	02	13	1		
	03	11	1	11	3
100	01	12	1		
	02	11	1		
	03	6	1	10	3
333	01	17	1		
	02	6	1		
	03	11	1	11	6
1000	01	13	1		
	02	10	1		
	03	8	1	10	3
3333	01	11	1		
	02	11	1		
	03	12	1	11	1
5000	01	11	1		
	02	13	1		
	03	11	1	12	1
Positive Control sodium azide 1.0 µg per plate[b]					
	01	556	1		
	02	535	1		
	03	559	1	550	13

[a]Background bacterial evaluation 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
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 11

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B1
Cells Seeded : 3.0 X 10^8
Date Plated : 12/12/96
Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	13	1		
	03	12	1	13	1
100	01	17	1		
	02	10	1		
	03	11	1	13	4
333	01	21	1		
	02	16	1		
	03	14	1	17	4
1000	01	17	1		
	02	25	1		
	03	20	1	21	4
3333	01	13	1		
	02	11	1		
	03	12	1	12	1
5000	01	16	1		
	02	14	1		
	03	16	1	15	1
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	91	1		
	02	116	1		
	03	128	1	112	19

[a]Background bacterial evaluation 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

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 12

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA97a
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl
Experiment No : B1
Cells Seeded : 3.1 X 10^8
Date Plated : 12/12/96
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	135	1		
	02	150	1		
	03	157	1	147	11
100	01	135	1		
	02	136	1		
	03	129	1	133	4
333	01	190	1		
	02	139	1		
	03	163	1	164	26
1000	01	162	1		
	02	166	1		
	03	173	1	167	6
3333	01	196	1		
	02	160	1		
	03	192	1	183	20
5000	01	180	1		
	02	188	1		
	03	193	1	187	7
Positive Control 9-aminoacridine 100 µg per plate					
	01	1934	1		
	02	2118	1		
	03	1682	1	1911	219

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 13

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA97a
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B1
Cells Seeded : 3.1 X 10^8
Date Plated : 12/12/96
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	235	1		
	02	230	1		
	03	205	1	223	16
100	01	220	1		
	02	240	1		
	03	254	1	238	17
333	01	204	1		
	02	233	1		
	03	146	1	194	44
1000	01	215	1		
	02	213	1		
	03	198	1	209	9
3333	01	192	1		
	02	221	1		
	03	219	1	211	16
5000	01	217	1		
	02	229	1		
	03	234	1	227	9
Positive Control 2-aminoanthracene 2.0 µg per plate					
	01	1230	1		
	02	1481	1		
	03	1662	1	1458	217

[a]Background bacterial evaluation 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

E. coli Mutagenicity Assay

Table 14

Test Article Id : H-22051
Study Number : G96CF43.502011 Experiment No : B1
Strain : WP2 *uvrA* (pKM101) Cells Seeded : 7.6 X 10^8
Liver Microsomes : None Date Plated : 12/12/96
Vehicle : water
Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	232	1		
	02	201	1		
	03	194	1	209	20
100	01	258	1		
	02	235	1		
	03	212	1	235	23
333	01	267	1		
	02	250	1		
	03	251	1	256	10
1000	01	236	1		
	02	279	1		
	03	271	1	262	23
3333	01	244	1		
	02	256	1		
	03	305	1	268	32
5000	01	273	1		
	02	313	1		
	03	272	1	286	23
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	1829	1		
	02	1771	1		
	03	1792	1	1797	29

[a]Background bacterial evaluation 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

E. coli Mutagenicity Assay

Table 15

Test Article Id	: H-22051		
Study Number	: G96CF43.502011	Experiment No	: B1
Strain	: WP2 uvrA (pKM101)	Cells Seeded	: 7.6 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 12/12/96
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	242	1		
	02	276	1		
	03	222	1	247	27
100	01	254	1		
	02	294	1		
	03	263	1	270	21
333	01	315	1		
	02	268	1		
	03	265	1	283	28
1000	01	305	1		
	02	286	1		
	03	310	1	300	13
3333	01	285	1		
	02	303	1		
	03	313	1	300	14
5000	01	296	1		
	02	303	1		
	03	293	1	297	5
Positive Control 2-aminoanthracene 10 µg per plate					
	01	1664	1		
	02	1591	1		
	03	1410	1	1555	131

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 16

Test Article Id : H-22051
Study Number : G96CF43.502011 Experiment No : B2
Strain : TA98 Cells Seeded : 1.1 X 10^8
Liver Microsomes : None Date Plated : 01/03/97
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	15	1		
	03	16	1	15	1
100	01	20	1		
	02	18	1		
	03	16	1	18	2
333	01	11	1		
	02	11	1		
	03	5	1	9	3
1000	01	7	1		
	02	10	1		
	03	16	1	11	5
3333	01	7	1		
	02	6	1		
	03	11	1	8	3
5000	01	4	1		
	02	2	1		
	03	11	1	6	5
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	138	1		
	02	152	1		
	03	122	1	137	15

[a]Background bacterial evaluation 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
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 17

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl
Experiment No : B2
Cells Seeded : 1.1 X 10^8
Date Plated : 01/03/97
Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	19	1		
	02	16	1		
	03	25	1	20	5
100	01	18	1		
	02	25	1		
	03	21	1	21	4
333	01	26	1		
	02	19	1		
	03	35	1	27	8
1000	01	20	1		
	02	13	1		
	03	16	1	16	4
3333	01	18	1		
	02	13	1		
	03	23	1	18	5
5000	01	6	1		
	02	7	1		
	03	14	1	9	4
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	746	1		
	02	824	1		
	03	656	1	742	84

[a]Background bacterial evaluation 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
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 18

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA100
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B2
Cells Seeded : 1.5×10^8
Date Plated : 01/03/97
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	134	1		
	02	134	1		
	03	93	1	120	24
100	01	121	1		
	02	131	1		
	03	82	1	111	26
333	01	121	1		
	02	116	1		
	03	112	1	116	5
1000	01	104	1		
	02	100	1		
	03	107	1	104	4
3333	01	134	1		
	02	117	1		
	03	125	1	125	9
5000	01	103	1		
	02	87	1		
	03	99	1	96	8
Positive Control sodium azide 1.0 µg per plate					
	01	699	1		
	02	696	1		
	03	660	1	685	22

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 19

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA100
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl
Experiment No : B2
Cells Seeded : 1.5 X 10^8
Date Plated : 01/03/97
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	139	1		
	02	117	1		
	03	121	1	126	12
100	01	129	1		
	02	142	1		
	03	114	1	128	14
333	01	150	1		
	02	117	1		
	03	132	1	133	17
1000	01	147	1		
	02	133	1		
	03	136	1	139	7
3333	01	141	1		
	02	132	1		
	03	127	1	133	7
5000	01	134	1		
	02	137	1		
	03	156	1	142	12
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	1183	1		
	02	1112	1		
	03	1019	1	1105	82

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 20

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA1535
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B2
Cells Seeded : 2.8 X 10^8
Date Plated : 01/03/97

Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	15	1		
	03	14	1	13	2
100	01	11	1		
	02	18	1		
	03	14	1	14	4
333	01	8	1		
	02	8	1		
	03	11	1	9	2
1000	01	8	1		
	02	9	1		
	03	10	1	9	1
3333	01	11	1		
	02	2	1		
	03	6	1	6	5
5000	01	11	1		
	02	9	1		
	03	6	1	9	3
Positive Control sodium azide 1.0 µg per plate[b]					
	01	552	1		
	02	549	1		
	03	524	1	542	15

[a]Background bacterial evaluation 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

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 21

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B2
Cells Seeded : 2.8 X 10^8
Date Plated : 01/03/97
Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	12	1		
	02	11	1		
	03	9	1	11	2
100	01	15	1		
	02	11	1		
	03	9	1	12	3
333	01	12	1		
	02	20	1		
	03	17	1	16	4
1000	01	18	1		
	02	10	1		
	03	13	1	14	4
3333	01	11	1		
	02	8	1		
	03	16	1	12	4
5000	01	12	1		
	02	18	1		
	03	10	1	13	4
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	97	1		
	02	102	1		
	03	103	1	101	3

[a]Background bacterial evaluation 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

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 22

Test Article Id	: H-22051	Experiment No	: B2
Study Number	: G96CF43.502011	Cells Seeded	: 1.3 X 10^8
Strain	: TA97a	Date Plated	: 01/03/97
Liver Microsomes	: None		
Vehicle	: water	Counted by	: machine
Plating Aliquot	: 50 µl		

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	116	1		
	02	104	1		
	03	98	1	106	9
100	01	146	1		
	02	126	1		
	03	114	1	129	16
333	01	125	1		
	02	154	1		
	03	112	1	130	22
1000	01	127	1		
	02	132	1		
	03	121	1	127	6
3333	01	109	1		
	02	132	1		
	03	130	1	124	13
5000	01	127	1		
	02	130	1		
	03	142	1	133	8
Positive Control 9-aminoacridine 100 µg per plate					
	01	2130	1		
	02	2283	1		
	03	876	1	1763	772

[a]Background bacterial evaluation 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

Salmonella Mutagenicity Assay

Table 23

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : TA97a
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B2
Cells Seeded : 1.3 X 10^8
Date Plated : 01/03/97

Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	147	1		
	02	155	1		
	03	142	1	148	7
100	01	146	1		
	02	146	1		
	03	159	1	150	8
333	01	140	1		
	02	168	1		
	03	162	1	157	15
1000	01	163	1		
	02	163	1		
	03	180	1	169	10
3333	01	154	1		
	02	155	1		
	03	160	1	156	3
5000	01	145	1		
	02	146	1		
	03	165	1	152	11
Positive Control 2-aminoanthracene 2.0 µg per plate					
	01	2068	1		
	02	2052	1		
	03	2126	1	2082	39

[a]Background bacterial evaluation 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

E. coli Mutagenicity Assay

Table 24

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : WP2 uvrA (pKM101)
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 50 µl

Experiment No : B2
Cells Seeded : 4.5 X 10^8
Date Plated : 01/03/97
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	164	1		
	02	145	1		
	03	150	1	153	10
100	01	192	1		
	02	144	1		
	03	175	1	170	24
333	01	182	1		
	02	194	1		
	03	193	1	190	7
1000	01	169	1		
	02	158	1		
	03	199	1	175	21
3333	01	158	1		
	02	180	1		
	03	212	1	183	27
5000	01	205	1		
	02	146	1		
	03	170	1	174	30
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	1501	1		
	02	1585	1		
	03	1500	1	1529	49

[a]Background bacterial evaluation 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

E. coli Mutagenicity Assay

Table 25

Test Article Id : H-22051
Study Number : G96CF43.502011
Strain : WP2 uvrA (pKM101)
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 50 µl
Experiment No : B2
Cells Seeded : 4.5×10^8
Date Plated : 01/03/97
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	164	1		
	02	171	1		
	03	160	1	165	6
100	01	182	1		
	02	165	1		
	03	171	1	173	9
333	01	203	1		
	02	185	1		
	03	229	1	206	22
1000	01	174	1		
	02	173	1		
	03	207	1	185	19
3333	01	187	1		
	02	199	1		
	03	212	1	199	13
5000	01	251	1		
	02	202	1		
	03	192	1	215	32
Positive Control 2-aminoanthracene 10 µg per plate					
	01	1668	1		
	02	1690	1		
	03	1714	1	1691	23

[a]Background bacterial evaluation 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

Salmonella/E. coli Mutagenicity Assay
Summary of Results

Table 26

Test Article Id : H-22051
Study Number : G96CF43.502011 Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg)	TA98	TA100	TA1535	TA97a	WP2 uvrA (pKM101)
0.0	25 ± 6	145 ± 14	11 ± 3	147 ± 11	209 ± 20
100	35 ± 10	133 ± 21	10 ± 3	133 ± 4	235 ± 23
333	16 ± 4	134 ± 14	11 ± 6	164 ± 26	256 ± 10
1000	21 ± 5	142 ± 15	10 ± 3	167 ± 6	262 ± 23
3333	17 ± 5	129 ± 9	11 ± 1	183 ± 20	268 ± 32
5000	20 ± 1	122 ± 11	12 ± 1	187 ± 7	286 ± 23
Pos	132 ± 37	747 ± 13	550 ± 13	1911 ± 219	1797 ± 29

Liver Microsomes: Rat liver S9

Dose (µg)	TA98	TA100	TA1535	TA97a	WP2 uvrA (pKM101)
0.0	19 ± 5	142 ± 16	13 ± 1	223 ± 16	247 ± 27
100	26 ± 2	146 ± 19	13 ± 4	238 ± 17	270 ± 21
333	27 ± 2	152 ± 10	17 ± 4	194 ± 44	283 ± 28
1000	32 ± 3	152 ± 13	21 ± 4	209 ± 9	300 ± 13
3333	32 ± 7	136 ± 14	12 ± 1	211 ± 16	300 ± 14
5000	27 ± 4	152 ± 8	15 ± 1	227 ± 9	297 ± 5
Pos	734 ± 42	782 ± 23	112 ± 19	1458 ± 217	1555 ± 131

0.0 = Vehicle plating aliquot of 50 µl
Pos = Positive Control concentrations as specified in Materials and Methods section.

Salmonella/E. coli Mutagenicity Assay
Summary of Results

Table 27

Test Article Id : H-22051
Study Number : G96CF43.502011 Experiment No : B2

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg)	TA98	TA100	TA1535	TA97a	WP2 uvrA (pKM101)
0.0	15 ± 1	120 ± 24	13 ± 2	106 ± 9	153 ± 10
100	18 ± 2	111 ± 26	14 ± 4	129 ± 16	170 ± 24
333	9 ± 3	116 ± 5	9 ± 2	130 ± 22	190 ± 7
1000	11 ± 5	104 ± 4	9 ± 1	127 ± 6	175 ± 21
3333	8 ± 3	125 ± 9	6 ± 5	124 ± 13	183 ± 27
5000	6 ± 5	96 ± 8	9 ± 3	133 ± 8	174 ± 30
Pos	137 ± 15	685 ± 22	542 ± 15	1763 ± 772	1529 ± 49

Liver Microsomes: Rat liver S9

Dose (µg)	TA98	TA100	TA1535	TA97a	WP2 uvrA (pKM101)
0.0	20 ± 5	126 ± 12	11 ± 2	148 ± 7	165 ± 6
100	21 ± 4	128 ± 14	12 ± 3	150 ± 8	173 ± 9
333	27 ± 8	133 ± 17	16 ± 4	157 ± 15	206 ± 22
1000	16 ± 4	139 ± 7	14 ± 4	169 ± 10	185 ± 19
3333	18 ± 5	133 ± 7	12 ± 4	156 ± 3	199 ± 13
5000	9 ± 4	142 ± 12	13 ± 4	152 ± 11	215 ± 32
Pos	742 ± 84	1105 ± 82	101 ± 3	2082 ± 39	1691 ± 23

0.0 = Vehicle plating aliquot of 50 µl
Pos = Positive Control concentrations as specified in Materials and Methods section.

APPENDIX I

Historical Control Data

Historical Negative and Positive Control Values
1993 - 1995

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	20	7	4	52	27	8	4	65
	Pos	316	185	17	3045	1091	538	94	3506
TA100	Neg	137	25	67	268	156	27	50	323
	Pos	672	194	100	2054	1133	510	136	3682
TA1535	Neg	12	5	1	53	13	5	0	46
	Pos	503	206	17	3704	128	130	18	2153
TA97 and TA97a	Neg	131	24	67	176	174	35	101	262
	Pos	1019	534	382	2541	1024	406	376	1842
WP2 *uvr*A (pKM101)	Neg	242	57	91	420	268	61	113	425
	Pos	1918	506	557	3072	1469	469	540	2668

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

APPENDIX II

Study Protocol

QA 12 2 96
APPROVED 11/27/96

MA Study Number: G96CF43.502011

Bacterial Reverse Mutation Assay with an Independent Repeat Assay

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article 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 *uvrA* (pKM101) 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: **Haskell Laboratory for Toxicology and Industrial Medicine**
PO Box 50, Elkton Road
Newark, DE 19714

2.3 Representative: **Brian H. Mathison, Ph.D.**

2.4 Sponsor Project #: **MR-10850**

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: ~~H-21938~~ H-22051 BHM 11/19/96
To correct sample identification

3.2 Controls: Negative: Test article vehicle

Positive: 9-aminoacridine
2-aminoanthracene
methyl methanesulfonate
2-nitrofluorene
sodium azide

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample

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

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
Microbiological Associates, Inc.

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

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

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12/3/96

5.2 Proposed Experimental Completion Date: 1/17/97

5.3 Proposed Report Date: 1/31/97

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA97A as described by Ames *et al.* (1975) and Levin *et al.* (1982) and the *E. coli* tester strain WP2 *uvr*A (pKM101) as described by Green and Muriel (1976).

Genotype of the Strains Used for Mutagen Testing

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*D3052	*his*D6610	*trp*E	LPS	Repair	R-factor
TA1535	-	-	-	*rfa*	Δ*uvr*B	-
TA100	TA98	TA97A	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A (pKM101)	-	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, TA100 and TA97A 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 TA97A 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 *trpE* gene (Wilcox *et al.*, 1990). Tester strain WP2 *uvrA* (pKM101) has a deletion in the *uvrA* gene resulting in a deficient DNA excision-repair system. Tester strain WP2 *uvrA* (pKM101) also contains the pKM101 plasmid described above. 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, rather than frameshift 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 article will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA97A and WP2 *uvrA* (pKM101) with and without S9 activation. All dose levels of test article, negative controls and positive controls will be plated in triplicate.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to: deionized water (CAS 7732-18-5), dimethylsulfoxide (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, that permits preparation of the highest workable/soluble stock concentration, up to 500 mg/ml.

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 article assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA98, TA100, TA1535, TA97A and WP2 *uvrA* (pKM101) to negative controls and to at least eight concentrations of test article, 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. 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 following the incubation period. In the event that the test article cannot be delivered at a high enough concentration in an appropriate vehicle to be toxic or if test article precipitate is present on the plates

after incubation, the Sponsor will be consulted prior to selection of dose levels for the mutagenicity assay. In selecting dose levels for the mutagenicity assay the following guidelines will be employed. 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 articles, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test articles, the highest dose level will be selected in an attempt to yield precipitate at only the top one or two dose levels. The precipitate will be evaluated after the incubation period by visual examination without magnification. Doses will be selected such that precipitate does not interfere with scoring.

7.3 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This methodology 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 range should be changed due to such parameters as excessive cytotoxicity or precipitate.

7.4 Controls

7.4.1 Positive Controls

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

Positive Controls

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA100, TA1535	+	2-aminoanthracene	1.0
TA97A	+	2-aminoanthracene	2.0
WP2 *uvrA* (pKM101)	+	2-aminoanthracene	10
TA98	-	2-nitrofluorene	1.0
TA100, TA1535	-	sodium azide	1.0
TA97A	-	9-aminoacridine	75
WP2 *uvrA* (pKM101)	-	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 article 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 article, test phase, dose level, tester strain and activation type as described in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.8 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article 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 article 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 article/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 4±2°C.

7.10 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article 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.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed for all tester strains by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed for tester strains TA98, TA100, TA1535 and TA97A by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98, TA100 and TA97A by demonstrating resistance to ampicillin.

On the day of use in the mutagenicity assay, the *E. coli* tester strain cultures will be checked for the presence of the *uvr*A mutation by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strain WP2 *uvr*A (pKM101) by demonstrating resistance to ampicillin.

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 *uvrB* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvrA* 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, TA100, TA97A and WP2 *uvrA* (pKM101) 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; TA97A, 80 - 240; WP2 *uvrA* (pKM101), 150 - 380.

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.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three 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 fewer 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 article 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 article as specified below:

9.1 Strain TA1535

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 three times the mean negative control value (vehicle).

9.2 Strains TA98, TA100, TA97A and WP2 *uvrA* (pKM101)

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 two times the mean negative control value (vehicle).

In consultation with the Sponsor, negative results may be confirmed as needed and equivocal results may be clarified by further testing using modified experimental conditions.

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 article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article 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

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guidelines 471 and 472 (Genetic Toxicology: Bacterial Reverse Mutation Assay), Revised Draft Document, September 1995 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft, July 18, 1995.

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

Will this study be submitted to a regulatory agency? yes If so, to which agency or agencies? EPA/TSCA, OECD, MAFF

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

13.0 REFERENCES

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 of Technical Requirements for Registration of Pharmaceuticals for Human Use, Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft, July 18, 1995.

Levin, D.E., Yamasaki, E. and Ames, B.N. (1982). A new *Salmonella* tester strain, TA97, for the detection of frameshift mutagens. A run of cytosines as a mutational hot-spot. Mutation Research 94:315-330.

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 Guidelines 471 and 472 (Genetic Toxicology: Bacterial Reverse Mutation Assay). Revised Draft Document, September 1995.

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.

14.0 APPROVAL

11/19/96
SPONSOR REPRESENTATIVE DATE

BRIAN M. MATHISON
(Print or Type Name)

Valentine C. Wagner, III 11/27/96
STUDY DIRECTOR DATE