AlliedSignal

AlliedSignal Inc.
P.O. Box 1057
Morristown, NJ 07962-1057

(1)

PDCN: 88940000088

December 21, 1994

93 JAN -3 PM 3:05
RECEIVED

Document Processing Center (7404)
Attn: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
Washington, DC 20460-0001

(C) Contains No CBI

RE: 8EHQ-1293-12803

Following up to my letter of November 21st and in response to your referenced correspondence, please find the attached final reports:

1. *Salmonella/Escherichia Coli* Mutagenicity Assay - FLEXAD (408-93C)

2. *In Vitro* Mammalian Cytogenetic Test Using Human Peripheral Lymphocytes and *In Vitro* Mammalian Cytogenetic Test Using Human Peripheral Lymphocytes: Confirmatory Assay.

The test material referenced in the reports, FLEXAD (408-93C) is the working name for the material identified in the original 8(e) submission: 3-Glycidoxypropylmethyldiethoxysilane (CAS#: 2897-60-1.)

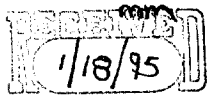
If you need to contact me, I can be reached at 201-455-2733.

Sincerely,

ORIGINAL

Daniel Levine

Daniel Levine
Director - Product Safety and Integrity



FINAL REPORT

Study Title

IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES
and
IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES: CONFIRMATORY ASSAY

Test Article

FLEXAD (408-93C)

Authors

Donald L. Putman, Ph.D.
Patrick T. Curry, Ph.D.
Elizabeth H. Schadly, B.S.

Study Completion Date

October 27, 1994

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, Maryland 20850

Laboratory Study Numbers

TD894.392005/392006

Sponsor Protocol/Project Number

93055/TOX-062A

Sponsor

ALLIED-SIGNAL INC.
Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

TOX Computer Entry No.
TOX-62A-93-4 11/3/94

MICROBIOLOGICAL ASSOCIATES, INC.

STATEMENT OF COMPLIANCE

The cytogenetic studies, *In Vitro* Mammalian Cytogenetic Test Using Human Peripheral Lymphocytes, TD894.392005 and *In Vitro* Mammalian Cytogenetic Test Using Human Peripheral Lymphocytes: Confirmatory Assay, TD894.392006, were conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

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

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

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

Donald L. Putman, Ph.D.
Study Director

10-27-94
Date

QUALITY ASSURANCE STATEMENT

Study Title: IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES and IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES: CONFIRMATORY ASSAY

Study Number: TD894.392005 and TD894.392006

Study Director: Donald L. Putman, Ph.D.

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, 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. The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 10/11/93 - 10/11/93, TO STUDY DIR 10/11/93 TO MGMT 10/11/93
PHASES: PROTOCOL REVIEW

INSPECT ON 10/11/93 - 10/11/93, TO STUDY DIR 10/11/93 TO MGMT 10/11/93
PHASES: PROTOCOL REVIEW

INSPECT ON 10/15/93 - 10/15/93, TO STUDY DIR 10/15/93 TO MGMT 10/18/93
PHASES: COLCEMID TREATMENT OF ACTIVATED SYSTEM

INSPECT ON 12/27/93 - 12/27/93, TO STUDY DIR 12/29/93 TO MGMT 01/04/94
PHASES: CODING OF THE SLIDES

INSPECT ON 01/31/94 - 02/01/94, TO STUDY DIR 02/01/94 TO MGMT 02/02/94
PHASES: DRAFT REPORT

INSPECT ON 10/03/94 - 10/03/94, TO STUDY DIR 10/03/94 TO MGMT 10/27/94
PHASES: DRAFT REPORT 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 — Oct 27, 1994

Claire L. Courtemanche
Quality Assurance Unit

Date

IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES
and
IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES: CONFIRMATORY ASSAY

FINAL REPORT

Sponsor: **ALLIED-SIGNAL INC.**
Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

Authorized Representative: **Gary Roy, M.B.A.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **FLEXAD (408-93C)**

MA Study No.: **TD894.392005/392006**

Sponsor Protocol/Project No.:**93051/TOX-062A**

Test Article Description: **Clear amber liquid**

Storage Conditions: **Room temperature;**
Protected from light

Test Article Receipt: **October 1, 1993**

Study Initiation: **October 8, 1993**

Study Director: Donald L. Putman 10-27-94
Donald L. Putman, Ph.D. Date

Associate Study Director: Patrick T. Curry 10-27-94
Patrick T. Curry, Ph.D. Date

Laboratory Supervisor: Elizabeth H. Schadly 10/27/94
Elizabeth H. Schadly, B.S. Date

TABLE OF CONTENTS

Page

Summary 6

Purpose 8

Characterization of Test and Control Articles 8

Materials and Methods 8

Results and Discussion 12

Conclusion 15

Data Tables 16

References 24

Appendix I: Historical Control Data 25

Appendix II: Study Protocols 27

SUMMARY

The test article, FLEXAD (408-93C), was tested in the *in vitro* mammalian cytogenetic test using human peripheral lymphocytes. The initial assay was conducted both in the absence and presence of an Aroclor-induced S-9 activation system at dose levels of 0.5, 1.5, 5, 15, 50, 150, 500, 1500, and 5000 μg/ml. The test article was soluble in solvent at stock concentration 500 mg/ml and was soluble in treatment medium at all concentrations tested. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment. Toxicity, as measured by mitotic inhibition, was approximately 56% at the highest dose level evaluated for chromosome aberrations in the nonactivated study, 150 μg/ml. In the S-9 activated study, toxicity was approximately 64% at the highest dose level evaluated for chromosome aberrations, 1500 μg/ml. Excessive toxicity (i.e., complete mitotic inhibition) precluded the analysis of test concentrations above 150 μg/ml in the nonactivated system and at 5000 μg/ml in the S-9 activated system. A statistically significant increase in structural chromosome aberrations was observed in both the nonactivated and S-9 activated test systems at concentrations 150 and 1500 μg/ml, respectively ($p \leq 0.01$, Fishers exact test) at the 20 hour harvest time. The Cochran-Armitage test was positive for a dose response in both the nonactivated and the S-9 activated test systems ($p \leq 0.05$). No significant increases in numerical aberrations were observed at any treatment level in the nonactivated or S-9 activated test systems.

Based on the results of the initial assay, a confirmatory assay was conducted in the absence of a metabolic activation system at dose levels of 6.25, 12.5, 25, 50, 100 and 200 μg/ml and in the presence of an Aroclor-induced S-9 metabolic activation system at dose levels of 47, 94, 188, 375, 750, and 1500 μg/ml. The test article was soluble in solvent and treatment medium at all concentrations tested. Metaphase cells were collected for microscopic evaluation at 20 and 44 hours after the initiation of treatment. Toxicity, measured by mitotic inhibition, was approximately 42% and 24% at the highest dose level evaluated for chromosome aberrations in the nonactivated studies, 200 μg/ml, at the 20 and 44 hour harvests, respectively. In the nonactivated study, concentrations of 6.25 and 12.5 μg/ml were tested but not required for microscopic analysis. In the S-9 activated studies, toxicity was approximately 62% and 60% at the highest dose level evaluated for chromosome aberrations, 1500 μg/ml, at the 20 and 44 hour harvests, respectively. Dose levels 47 and 94 μg/ml were tested but not required for microscopic analysis. A statistically significant increase in structural chromosome aberrations was observed in the nonactivated 44 hour harvest only at concentrations 100 and 200 μg/ml ($p \leq 0.05$ and $p \leq 0.01$ for the 100 and 200 μg/ml concentrations, respectively, Fisher's exact test). The Cochran-Armitage test was positive for a dose response in the nonactivated 44 hour harvest time ($p \leq 0.05$). No statistically significant increases in structural aberrations were observed in the nonactivated 20 hour harvest or S-9 activated test system at the 20 or 44 hour harvest times ($p > 0.05$, Fisher's exact test). No significant increases in numerical aberrations were observed at any treatment level in the

nonactivated or S-9 activated test systems, regardless of harvest time. Based on the nonreproducibility of a statistically significant increase at a single dose level in the S-9 activated study, FLEXAD (408-93C) was concluded to be negative in the S-9 activated test system in the *in vitro* mammalian cytogenetics test using human peripheral lymphocytes. FLEXAD (408-93C) was concluded to be positive in the nonactivated test system in the *in vitro* mammalian cytogenetics test using human peripheral lymphocytes.

PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral lymphocytes.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, FLEXAD (408-93C), was received by Microbiological Associates, Inc. on October 1, 1993 and was assigned the code number D894. The test article was characterized by the Sponsor as a clear amber liquid that should be stored in a temperature-monitored room 65°-85°F protected from moisture with no expiration date provided

Upon receipt, the test article was described as a clear amber liquid and was stored at room temperature, protected from exposure to light. At the time of use, the test article was dissolved in acetone, CAS 67-64-1, lots 920807 and 920961, obtained from Fisher Chemical Company.

Mitomycin C (MMC), lot 72H0197, was obtained from Sigma Chemical Company, was dissolved and diluted in deionized, distilled water to stock concentrations of 12.5 and 25 μg/ml. Cyclophosphamide (CP), lot 72H0088, was obtained from Sigma Chemical Company, and was dissolved and diluted in deionized, distilled water to stock concentrations of 2.5 and 5 mg/ml.

MATERIALS AND METHODS

Test System

Peripheral blood lymphocytes were obtained from a single healthy adult male with no recent history of either radiotherapy, viral infection or the administration of drugs.

Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 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 S-9 was batch prepared and stored at -70°C until used. Each bulk preparation of S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μl S-9 per milliliter medium (RPMI 1640 serum-free medium supplemented with 100 units penicillin and 100 μg streptomycin/ml, 2 mM L-glutamine, and 1% PHA).

Definitive Assays (Initial and Confirmatory)

The initial assay was performed by exposing replicate cultures of human peripheral blood lymphocytes (HPBL) to a minimum of nine concentrations of the test article as well as positive and negative controls. In the initial assay, the dividing cells were harvested at a single time point, approximately 20 hours from the initiation of treatment (1.5 times normal cell cycle). The highest dose level demonstrating at least 50% reduction in mitotic index and three lower doses were evaluated microscopically for structural chromosome aberrations.

The confirmatory assay was performed using a minimum of four concentrations of the test article. These concentrations were selected based on the findings of the initial assay. For the independent repeat assay, the dividing cells were harvested at two time points, approximately 20 hours from the initiation of treatment (1.5 times cell cycle) and 24 hours later.

For the chromosome aberration assays, 0.6 ml heparinized blood was inoculated into centrifuge tubes containing 9.4 ml complete medium supplemented with 1% PHA. The tubes were incubated at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air for 44-48 hours. The addition of 0.6 ml of heparinized blood to centrifuge tubes containing 9.4 ml of complete medium represents a deviation from the protocol. The protocol requires the addition of 0.6 ml of blood to 10 ml of complete medium. This deviation from protocol was documented in the raw data with a deviation report and was concluded by the Study Director to have no significant effect on the conclusions or the quality of the study. Treatment was carried out by refeeding with approximately 10 ml fresh complete medium or S-9 reaction mixture to which was added 100 μl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture was also included.

In the non-activated studies, the cells were exposed for 20 or 44 hours at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air. Two hours prior to the scheduled cell harvest at 20 or 44 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 μg/ml.

In the S-9 activated studies, the cells were exposed for 4 hours at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with PBS, refed with complete medium containing 1% PHA and returned to the incubator for an additional 16 hours for the first harvest and 40 hours for the delayed harvest. Two hours prior to the

scheduled cell harvest at 20 or 44 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 μg/ml.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the activated and nonactivated studies by centrifugation. The cells were collected by centrifugation at approximately 1200 rpm for about 5 minutes. The cell pellet was resuspended in 5 ml 0.075 M KCl and incubated at 37±1°C for 20 minutes. At the end of the KCl treatment and immediately prior to centrifuging, the cells were gently mixed and approximately 0.5 ml of fixative (methanol:glacial acetic acid, 3:1 v/v) was added to each tube. The cells were collected by centrifugation, the supernatant aspirated, and the cells were fixed with two washes with approximately 3-5 ml fixative and stored in fixative overnight or longer at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 1200 rpm for 5 minutes, the supernatant fluid was aspirated, and the cells were resuspended in 1 ml cold fresh fixative. The cells were collected by centrifugation and the supernatant aspirated, leaving 0.1 to 0.3 ml fixative above the cell pellet. One to two drops of cell suspension were dropped onto a moist glass slide and allowed to air dry overnight. Two slides were made for each treatment or control flask. Slides were identified by the study number, dose level, activation condition, harvest time, replicate tube designation and date prepared. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate treatment condition) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ($\geq$10 aberrations) also were recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index

was recorded as the percentage of cells in mitosis per 500 cells counted. The percent polyploid or endoreduplicated cells per 100 metaphase cells was determined for each culture.

Controls

Mitomycin C was used as the positive control in the non-activated study at final concentrations of 0.13 and 0.25 μg/ml. Cyclophosphamide was used as the positive control in the S-9 activated study at final concentrations of 25 and 50 μg/ml. For both positive controls the highest dose with sufficient scorable metaphase cells was selected for analysis. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S-9 reaction mixture was used in the untreated control.

Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for the initial and confirmatory assays. The number and types of aberrations found are presented for each treatment group. The percentage of structurally or numerically damaged cells (percent aberrant cells) in the total population of cells examined was calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) was also calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). Treatment groups significantly elevated above the solvent control group at $p \leq 0.01$ will be noted. A reproducible and significant increase at a single dose level will be considered positive.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

All raw data, draft and final reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Initial Assay

HPBL cells were first exposed to the following concentrations of FLEXAD (408-93C): 0.5, 1.5, 5, 15, 50, 150, 500, 1500, and 5000 μg/ml in both the absence and presence of an Aroclor-induced S-9 activation system. The test article was soluble at stock concentration 500 mg/ml in acetone and was soluble in treatment medium at all concentrations tested in both the nonactivated and S-9 activated studies. The osmolality and pH of the highest concentration tested, 5000 μg/ml, was 371 mOsm/kg and approximately 7.5, respectively.

The activity of FLEXAD (408-93C) in the induction of chromosome aberrations in HPBL cells was tested following a 20 hour exposure in the absence of an exogenous source of metabolic activation. According to the protocol, the highest dose level with at least 50% mitotic inhibition and three lower dose levels were selected for microscopic analysis for chromosome aberrations. The findings of the cytogenetic analysis are presented by treatment flask in Table 1 and summarized by group in Table 7. At the highest test concentration evaluated for chromosome aberrations, 150 μg/ml, the mitotic index was 56% reduced relative to the solvent control. Complete mitotic inhibition precluded the analysis of test concentrations 500 μg/ml or higher. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at the highest concentration evaluated for chromosome aberrations, 150 μg/ml dose level ($p \leq 0.01$, Fisher's exact test). A positive dose trend was observed in this test system ($p \leq 0.05$, Cochran-Armitage test). The percentage of cells with numerical aberrations was not significantly increased above that of the solvent control at the 20 hour harvest ($p > 0.05$, Fisher's exact test) regardless of treatment level. The percentage of damaged cells in the MMC group was 12% ($p \leq 0.01$, Fisher's exact test).

The activity of FLEXAD (408-93C) in the induction of chromosome aberrations in HPBL cells was tested using a 4 hour exposure in the presence of an exogenous source of metabolic activation followed by a 16 hour recovery period. According to protocol, the highest dose level with at least 50% reduction in mitotic index and three lower dose levels were selected for microscopic analysis for chromosome aberrations. The findings of the cytogenetic analysis are presented by treatment flask in Table 4 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 1500 μg/ml, the mitotic index was 64% reduced relative to the solvent control. Complete mitotic inhibition precluded analysis of dose level 5000 μg/ml. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at the highest concentration evaluated for chromosome aberrations, 1500 μg/ml ($p \leq 0.01$, Fisher's exact test). A positive dose trend was observed in this test system ($p \leq 0.05$, Cochran-Armitage test). The percentage of cells with numerical aberrations was not significantly increased above that of the solvent control at the 20 hour harvest ($p > 0.05$, Fisher's exact test) regardless of treatment level. The percentage of damaged cells in the CP group was 15.5% ($p \leq 0.01$, Fisher's exact test).

Confirmatory Assay

Based on the results of the initial assay, dose levels of 6.25, 12.5, 25, 50, 100, and 200 μg/ml were selected for further study in the nonactivated portion of the confirmatory assay using 20 hour and 44 hour exposure periods. In the S-9 activated study, dose levels 47, 94, 188, 375, 750, and 1500 μg/ml were selected for further study in the confirmatory assay using a 4 hour exposure period with 20 and 44 hour harvest times. The test article was soluble in solvent and treatment medium at all dose levels tested. The osmolalities of the highest concentrations tested, 200 and 1500 μg/ml were 426 and 404 mOsm/kg, respectively. The pH of the highest concentrations tested, 200 and 1500 μg/ml, was approximately 7.5.

The findings of the cytogenetic analysis for groups tested in the absence of an exogenous source of metabolic activation are presented by treatment flask in Tables 2 and 3 and summarized by group in Table 8. At the highest test concentration evaluated for chromosome aberrations, 200 μg/ml, the mitotic indices were 42% and 24% reduced relative to the solvent control at the 20 hour and 44 hour harvests, respectively. Dose levels 6.25 and 12.5 μg/ml were tested as a safeguard against toxicity but were not required for microscopic evaluation. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at test concentrations 100 and 200 μg/ml in the 44 hour harvest ($p \leq 0.05$ and $p \leq 0.01$, respectively, Fisher's exact test). There was also a positive dose trend in this test system ($p \leq 0.05$, Cochran-Armitage test). The percentage of cells with numerical aberrations was not significantly increased above that of the solvent control at either the 20 of 44 hour harvest times ($p > 0.05$, Fisher's exact test). The percentages of damaged cells in the MMC groups were 10% and 38% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour harvests, respectively.

The findings of the cytogenetic analysis for groups tested using a 4 hour exposure in the presence of an exogenous source of metabolic activation followed by a 16 or 40 hour recovery period are presented by treatment flask in Tables 5 and 6 and summarized by group in Table 8. At the highest test concentration evaluated for chromosome aberrations, 1500 μg/ml, the mitotic indices were 62% and 60% reduced relative to the solvent control in the 20 and 44 hour harvests, respectively. Dose levels 47 and 94 μg/ml were tested as a safeguard against toxicity but were not required for microscopic evaluation. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control at either the 20 or 44 hour harvest times ($p > 0.05$, Fisher's exact test). The percentage of cells with numerical aberrations was not significantly increased above that of the solvent control at either the 20 or 44 hour harvest times ($p > 0.05$, Fisher's exact test). The percentages of damaged cells in the CP groups were 21% and 23.5% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour harvests, respectively.

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, and the nonreproducibility of a statistically significant increase at a single dose level in the S-9 activated study, FLEXAD (408-93C) was concluded to be negative in the S-9 activated test system in the *in vitro* mammalian cytogenetics test using human peripheral blood lymphocytes. FLEXAD (408-93C) was concluded to be positive in the nonactivated test system in the *in vitro* mammalian cytogenetics test using human peripheral blood lymphocytes.

TABLE 1
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	3.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.8	100	0	0	0	0	0	0	0	0	0	0.000
Acetone	A	2.2	100	0	0	2	0	0	0	0	0	0	0.000
	B	2.8	100	0	0	0	0	0	0	0	0	0	0.000
FLEXAD (408-93C) 5 µg/ml	A	3.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.8	100	0	0	0	0	0	0	0	0	0	0.000
15 µg/ml	A	4.2	100	0	2	0	2	0	0	0	0	0	0.020
	B	4.0	100	0	0	0	0	0	0	0	0	0	0.000
50 µg/ml	A	2.4	100	0	1	0	1	0	0	0	0	0	0.010
	B	2.2	100	0	2	0	2	0	0	0	0	0	0.020
150 µg/ml	A	0.4	100	0	8	1	8	1	0	0	0	0	0.090
	B	1.8	100	0	10	1	12	0	0	0	0	0	0.120
MMC 0.13 µg/ml	A	2.0	100	0	10	0	14	1	0	0	0	0	0.150
	B	2.6	100	0	14	0	14	3	0	0	0	0	0.170

[1]HPBL cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 2
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

CONFIRMATORY ASSAY: 20 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	2.6	100	1	0	2	0	0	0	0	0	0	0.000
	B	2.0	100	0	0	0	0	0	0	0	0	0	0.000
Acetone	A	2.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.4	100	1	0	0	0	0	0	0	0	0	0.000
FLEXAD (408-93C) 25 µg/ml	A	1.4	100	0	1	0	1	0	0	0	0	0	0.010
	B	1.6	100	1	0	0	0	0	0	0	0	0	0.000
50 µg/ml	A	2.2	100	2	0	1	0	0	0	0	0	0	0.000
	B	2.4	100	2	0	1	0	0	0	0	0	0	0.000
100 µg/ml	A	1.6	100	0	0	1	0	0	0	0	0	0	0.000
	B	1.4	100	0	2	2	3	0	0	0	0	0	0.030
200 µg/ml	A	1.4	100	1	0	0	0	0	0	0	0	0	0.000
	B	1.4	100	1	1	0	1	0	1	0	0	0	0.020
MMC 0.13 µg/ml	A	1.0	100	0	11	3	8	6	1	0	0	0	0.150
	B	1.2	100	2	9	0	4	2	4	0	0	0	0.100

[1]HPBL cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 3
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

CONFIRMATORY ASSAY: 44 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	6.0	100	2	0	0	0	0	0	0	0	0	0.000
	B	6.4	100	3	0	0	0	0	0	0	0	0	0.000
Acetone	A	2.4	100	1	0	1	0	0	0	0	0	0	0.000
	B	3.4	100	1	1	0	2	0	0	0	0	0	0.020
FLEXAD (408-93C) 25 µg/ml	A	3.6	100	0	0	1	0	0	0	0	0	0	0.000
	B	2.8	100	1	0	0	0	0	0	0	0	0	0.000
50 µg/ml	A	3.0	100	1	0	0	0	0	0	0	0	0	0.000
	B	3.2	100	0	0	0	0	0	0	0	0	0	0.000
100 µg/ml	A	2.6	100	1	4	4	3	0	1	0	0	0	0.040
	B	3.0	100	0	3	0	3	0	1	0	0	0	0.040
200 µg/ml	A	2.6	100	2	12	2	13	0	3	0	0	0	0.160
	B	1.8	100	1	20	12	32	3	9	0	0	0	0.440
MMC 0.13 µg/ml	A	3.0	100	2	41	13	64	32	21	0	1	2	1.380
	B	2.2	100	2	35	13	62	17	13	0	0	0	0.920

[1]HPBL cells were treated for 44 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 4
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	2.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.6	100	0	0	0	0	0	0	0	0	0	0.000
Acetone	A	2.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.0	100	0	0	0	0	0	0	0	0	0	0.000
FLEXAD (408-93C) 50 µg/ml	A	2.4	100	0	1	0	0	1	0	0	0	0	0.010
	B	2.4	100	0	0	0	0	0	0	0	0	0	0.000
150 µg/ml	A	2.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.0	100	0	1	0	1	0	0	0	0	0	0.010
500 µg/ml	A	3.8	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.0	100	0	0	0	0	0	0	0	0	0	0.000
1500 µg/ml	A	0.6	100	0	15	0	19	4	0	0	0	0	0.230
	B	1.0	100	0	18	1	26	0	1	0	0	0	0.270
CP 25 µg/ml	A	0.6	100	0	16	0	22	2	0	0	0	0	0.240
	B	0.4	100	0	15	1	21	1	0	0	0	0	0.220

[1]HPBL cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 5
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

CONFIRMATORY ASSAY: 20 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	2.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.2	100	0	0	0	0	0	0	0	0	0	0.000
Acetone	A	2.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.2	100	2	0	0	0	0	0	0	0	0	0.000
FLEXAD (408-93C0													
188 µg/ml	A	1.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.2	100	0	1	0	1	0	0	0	0	0	0.010
375 µg/ml	A	1.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	0.6	100	0	0	0	0	0	0	0	0	0	0.000
750 µg/ml	A	1.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0	0.000
1500 µg/ml	A	0.6	100	0	1	0	1	0	0	0	0	0	0.010
	B	1.0	100	1	2	0	2	0	0	0	0	0	0.020
CP	A	0.6	100	0	20	0	35	10	3	0	1	0	0.490
25 µg/ml	B	0.2	100	0	22	0	32	0	0	0	0	0	0.320

[1]HPBL cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 6
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH FLEXAD (408-93C) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

CONFIRMATORY ASSAY: 44 HOUR HARVEST

Treatment[1]	Flask	Mitotic Index[2]	Cells Scored	Cells with Aberrations[3] (%)		Number of Structural Aberrations						Severely Damaged Cells[6]	Average Structural Aberrations Per Cell[3,7]
						Chromatid-type[4]			Chromosome-type[5]				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	5.6	100	1	0	1	0	0	0	0	0	0	0.000
	B	8.2	100	1	1	0	1	0	0	0	0	0	0.010
Acetone	A	7.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	9.2	100	0	0	0	0	0	0	0	0	0	0.000
FLEXAD (408-93C) 188 µg/ml	A	5.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.4	100	0	0	0	0	0	0	0	0	0	0.000
375 µg/ml	A	4.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.6	100	0	0	0	0	0	0	0	0	0	0.000
750 µg/ml	A	4.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	5.2	100	0	3	0	3	0	0	0	0	0	0.030
1500 µg/ml	A	3.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.4	100	0	0	1	0	0	0	0	0	0	0.000
CP 25 µg/ml	A	3.4	100	0	24	0	43	7	1	0	1	0	0.520
	B	3.0	100	0	23	0	37	6	1	1	0	0	0.450

[1]HPBL cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Numerical aberrations includes polyploid cells and endoreduplications; Cells with structural aberrations excludes cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 7
SUMMARY
INITIAL ASSAY

Treatment	S-9 Activation	Harvest Time (Hours)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)	
						Numerical	Structural
Untreated	-	20	3.4	200	0.000 ± 0.000	0.0	0.0
Acetone	-	20	2.5	200	0.000 ± 0.000	0.0	0.0
FLEXAD (408-93C)							
5 µg/ml	-	20	3.5	200	0.000 ± 0.000	0.0	0.0
15 µg/ml	-	20	4.1	200	0.010 ± 0.100	0.0	1.0
50 µg/ml	-	20	2.3	200	0.015 ± 0.122	0.0	1.5
150 µg/ml	-	20	1.1	200	0.105 ± 0.353	0.0	9.0**
MMC 0.13 µg/ml	-	20	2.3	200	0.160 ± 0.475	0.0	12.0**
Untreated	+	20	3.0	200	0.000 ± 0.000	0.0	0.0
Acetone	+	20	2.2	200	0.000 ± 0.000	0.0	0.0
FLEXAD (408-93C)							
50 µg/ml	+	20	2.4	200	0.005 ± 0.071	0.0	0.5
150 µg/ml	+	20	2.8	200	0.005 ± 0.071	0.0	0.5
500 µg/ml	+	20	2.9	200	0.000 ± 0.000	0.0	0.0
1500 µg/ml	+	20	0.8	200	0.250 ± 0.663	0.0	16.5**
CP 25 µg/ml	+	20	0.5	200	0.230 ± 0.591	0.0	15.5**

[1]Severely damaged cells were counted as 10 aberrations.
[2] **, p≤0.01; Fisher's exact test.

TABLE 8
SUMMARY
CONFIRMATORY ASSAY

Treatment	S-9 Activation	Harvest Time (Hours)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%) Numerical	Structural
Untreated	-	20	2.3	200	0.000 ± 0.000	0.5	0.0
Acetone	-	20	2.4	200	0.000 ± 0.000	0.5	0.0
FLEXAD (408-93C)							
25 µg/ml	-	20	1.5	200	0.005 ± 0.071	0.5	0.5
50 µg/ml	-	20	2.3	200	0.000 ± 0.000	2.0	0.0
100 µg/ml	-	20	1.5	200	0.015 ± 0.158	0.0	1.0
200 µg/ml	-	20	1.4	200	0.010 ± 0.141	1.0	0.5
MMC 0.13 µg/ml	-	20	1.1	200	0.125 ± 0.400	1.0	10.0**
Untreated	-	44	6.2	200	0.000 ± 0.000	2.5	0.0
Acetone	-	44	2.9	200	0.010 ± 0.141	1.0	0.5
FLEXAD (408-93C)							
25 µg/ml	-	44	3.2	200	0.000 ± 0.000	0.5	0.0
50 µg/ml	-	44	3.1	200	0.000 ± 0.000	0.5	0.0
100 µg/ml	-	44	2.8	200	0.040 ± 0.221	0.5	3.5*
200 µg/ml	-	44	2.2	200	0.300 ± 0.902	1.5	16.0**
MMC 0.13 µg/ml	-	44	2.6	200	1.150 ± 1.883	2.0	38.0**
Untreated	+	20	2.4	200	0.000 ± 0.000	0.0	0.0
Acetone	+	20	2.1	200	0.000 ± 0.000	1.0	0.0
FLEXAD (408-93C0							
188 µg/ml	+	20	1.9	200	0.005 ± 0.071	0.0	0.5
375 µg/ml	+	20	0.8	200	0.000 ± 0.000	0.0	0.0
750 µg/ml	+	20	1.5	200	0.000 ± 0.000	0.0	0.0
1500 µg/ml	+	20	0.8	200	0.015 ± 0.122	0.5	1.5
CP 25 µg/ml	+	20	0.4	200	0.405 ± 1.052	0.0	21.0**
Untreated	+	44	6.9	200	0.005 ± 0.071	1.0	0.5
Acetone	+	44	8.2	200	0.000 ± 0.000	0.0	0.0
FLEXAD (408-93C)							
188 µg/ml	+	44	4.7	200	0.000 ± 0.000	0.0	0.0
375 µg/ml	+	44	4.4	200	0.000 ± 0.000	0.0	0.0
750 µg/ml	+	44	4.6	200	0.015 ± 0.122	0.0	1.5
1500 µg/ml	+	44	3.3	200	0.000 ± 0.000	0.0	0.0
CP 25 µg/ml	+	44	3.2	200	0.485 ± 1.178	0.0	23.5**

[1]Severely damaged cells were counted as 10 aberrations.
[2]*, p≤0.05; **, p≤0.01; Fisher's exact test.

REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol 4. Plenum Press, New York.

Perry, P., and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

Appendix I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES

HISTORICAL CONTROL VALUES
1991-1993

NONACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	0.2%	0.3%	17.8%
Standard Deviation	0.4%	0.7%	9.8%
Range	0.0% to 2.0%	0.0% to 4.5%	2.5% to 49.0%

S-9 ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	0.3%	0.3%	11.8%
Standard Deviation	0.4%	0.6%	3.2%
Range	0.0% to 2.0%	0.0% to 3.0%	6.5% to 20.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone
[2]Positive control for nonactivated studies, mitomycin C, (MMC)
[3]Positive control for S-9 activated studies, cyclophosphamide, (CP)

APPENDIX II

Study Protocols

PROTOCOL AMENDMENT I

SPONSOR:	**ALLIED-SIGNAL INC.**
TEST ARTICLE I.D.:	**FLEXAD (408-93C)**
MA STUDY NO:	**TD894.392005 (Protocol No.: SPGT392005)**
SPONSOR PROJECT NO.:	**93051/TOX-062A**
PROTOCOL TITLE:	***IN VITRO* MAMMALIAN CYTOGETETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES**

---

1. **LOCATION:** Page 2, § 7.0

   AMENDMENT: The second sentence should read: "In the nonactivated test system, exposure will be continuous until harvest;..."

   REASON FOR THE AMENDMENT: To be consistent with section 7.7, second paragraph, for exposure time in the nonactivated assay.

APPROVALS:

STUDY DIRECTOR	3-18-94 DATE
SPONSOR REPRESENTATIVE	4-7-94 DATE
QUALITY ASSURANCE	3-15-94 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

PROTOCOL AMENDMENT II

SPONSOR: **ALLIED-SIGNAL INC.**

TEST ARTICLE I.D.: **FLEXAD (408-93C)**

MA STUDY NO: **TD894.392005 (Protocol No.: SPGT392005)**

SPONSOR PROJECT NO.: **93051/TOX-062A**

PROTOCOL TITLE: ***IN VITRO* MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES**

---

1. **LOCATION:** Protocol Appendix A, § I; page 1 of 2

AMENDMENT: The color of test article should read "clear and amber".

REASON FOR THE AMENDMENT: Sponsor request.

APPROVALS:

STUDY DIRECTOR	10-3-94 DATE
SPONSOR REPRESENTATIVE	10/10/94 DATE
Claire L Courtemanche QUALITY ASSURANCE	Oct 4, 1994 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

*MA Study Number: TD894.392005

IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES

APPROVED

1.0 PURPOSE

The purpose of this study is to test the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral lymphocytes.

2.0 SPONSOR

2.1 Name: ALLIED-SIGNAL INC.

2.2 Address: Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

2.3 Representative: Gary Roy, M.B.A.

* 2.4 Sponsor Protocol/Project No.: 93051/TOX-062A

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Sponsor Test Article ID/Sample No.: FLEXAD (408-93C)

3.2 Controls:
Solvent: Solvent vehicle
Negative: Untreated cells
Positive: Mitomycin C and cyclophosphamide

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.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic and Cellular Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Donald L. Putman, Ph.D.

*To be completed upon authorization

* 5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 10-12-93

5.2 Proposed Experimental Completion Date: 1-7-94

5.3 Proposed Audited Draft Report Date: 1-14-94

*To be completed upon authorization

6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from a single healthy adult volunteer without a history of either radiotherapy, viral infections or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing lymphocytes to a minimum of nine concentrations of the test article as well as positive and negative controls in duplicate cultures. In the nonactivated test system, exposure will be continuous up to the time of Colcemid treatment; in the S-9 activated system, exposure will be for 4 hours. The dividing cells will be harvested at a single time point, approximately 20 hours from the initiation of treatment (1.5 times normal cell cycle). The highest dose level with at least 50% mitotic inhibition and three lower doses will be evaluated microscopically for structural chromosome aberrations. The mutagenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Dose Levels

For the cytogenetics assay, lymphocytes will be exposed to at least nine concentrations of test article at half-log dilutions, the highest concentration being the limit of solubility but not to exceed 5 mg/ml for solids or 5 ul/ml for liquids. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition will also be measured.

7.2 Frequency and Route of Administration

Target cells will be treated for approximately 20 hours in the nonactivated system and for 4 hours in the S-9 activated system. Treatment will be achieved by incorporation of the test article-solvent mixture into the medium. This technique has demonstrated to be an effective method of exposure of mammalian cells in culture to concentrations of chemical.

7.3 Activation System

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The S-9 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 S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with cofactors to contain 2mM $MgCl_2$, 6 mM KCl, 1mM glucose-6-phosphate, 1mM NADP and 20 ul S-9 per ml serum free medium.

7.4 Controls

7.4.1 Untreated Controls

Untreated cells will be used as the untreated control.

7.4.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. The solvents compatible with this test system include water or medium, dimethylsulfoxide, acetone and ethanol. For solvents other than medium, the final concentration in treatment medium will not exceed 1%.

7.4.3 Positive Controls

Mitomycin C (MMC) will be used at two concentrations within 0.10-0.25 ug/ml as the positive control in the non-activated study. Cyclophosphamide (CP) will be used at two concentrations within 25-100 ug/ml as the positive control in the S-9 activated study. For each positive control chemical, a single concentration, the highest with sufficient scorable metaphase cells, will be selected for microscopic evaluation.

7.5 Preparation of Target Cells

Peripheral blood cells will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 ug streptomycin/ml) containing 1% phytohemagglutinin (PHA) by adding 0.6 ml heparinized blood to a centrifuge tube containing 10 ml complete medium with 1% PHA. The tubes will be incubated upright at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 44-48 hours.

7.6 Identification of Test System

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

7.7 Treatment of Target Cells

All treatments will be carried out in duplicate. About 46 hours after culture initiation, tubes will be refed with 10 ml complete medium for the nonactivated exposure or 10 ml of S-9 reaction mixture for the activated exposure to which will be added 100 ul of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture will also be included.

In the non-activated study, the cells will be exposed for 20 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Treatment will be continuous with Colcemid (0.1 ug/ml) present for the last two hours.

In the S-9 activated study, the cells will be exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium will be aspirated, the cells washed free of chemical and cultured for an additional 16 hours.

7.8 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after initiation of treatment. This time is selected to assure analysis of first division metaphase cells after initiation of test article treatment. Two hours prior to harvest, Colcemid will be added to the cultures at a final concentration of 0.1 ug/ml. The cells will be collected by centrifugation, treated with 0.075 M KCl, washed with two changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 4±2°C. To prepare slides, the cells will be collected by centrifugation, resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a wet slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

7.9 Scoring for Metaphase Aberrations

Slides will be scanned for scorable metaphases. The highest dose level with at least 50% reduction in mitotic index and three lower dose levels, if available, will then be coded for microscopic evaluation. Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted per culture. The percentage of polyploid or endoreduplicated cells per 100 metaphase cells will be determined for each culture.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water is used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented. The number and types of aberrations found will be presented for each treatment group. The percentage of structurally

damaged cells (percent aberrant cells) in the total population of cells examined will be calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) will also be calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells will be performed using the Fisher's exact test and reported for $p \leq 0.05$ and $p \leq 0.01$. Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage trend test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). Treatment groups significantly elevated above the solvent control group at $p \leq 0.01$ will be noted. A reproducible and significant increase at a single dose level will be considered positive.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data. Data will be presented in tabular form for the relative toxic effects of treatment, the number and types of aberrations observed, the frequency of cells with aberrations and the average aberrations per cell.

Results presented for the chromosome aberration assay will include:

- details of the protocol used for preparation of the microsomal enzymes
- dose levels at which the test article was tested
- dose response relationship, if applicable
- historical control data for negative or solvent controls
- evaluation of results
- Microbiological Associates report and study numbers, and Sponsor protocol/project numbers
- identification of test article by Sponsor test material ID and sample number

- observations of any insolubility, film or precipitate of test material and concentrations at which it occurred

- Good Laboratory Practices Compliance Statement, signed by the Study Director

- dates of Quality Assurance reviews of the study, including at least one "in process" inspection of the definitive assay, and of reports to management

- protocols, protocol appendices, and any amendments (signed by Sponsor, Testing Laboratory QA Unit and Study Director) shall be included as report appendices. Deviations from the protocol or Standard Operating Procedures not addressed by protocol amendment will be listed and their impact on the study noted

An audited draft report will be submitted to Sponsor for review and approval. Upon acceptance, five copies of the final report will be sent to Sponsor.

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates Inc. in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EEC Directive 79/831 Annex V, B.10 Mutagenicity (In Vitro Mammalian Cytogenetic Test), June, 1989; OECD Guideline 473 (Genetic Toxicology: In Vitro Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 798.5375 (In Vitro Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. A confirmatory assay will be required in order to comply with EEC guidelines.

No less than one in-lab audit will be conducted by the Testing Laboratory's QA Unit.

This study will be performed in compliance with the provisions of the US FDA Good Laboratory Practice Regulations (21 CFR 58), the US EPA GLP Regulations (40 CFR 160 and 40 CFR 792), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice.

This study may be submitted to the US EPA and therefore will be listed on the EPA Master Schedule List.

The Test Article will be returned to the Sponsor at the conclusion of the study. Samples of the Test Article dosing solutions are not required by the Sponsor and will be disposed of in accordance with all local, state and federal laws.

13.0 REFERENCES

Evans, H.J. 1976. Cytological methods for detecting chemical mutagens. In: Chemical Mutagens, Principles and Methods for their Detection, vol. 4. A. Hollaender (ed). Plenum Press, New York, NY.

Perry, P. and Wolff, S. 1974. New Giemsa method for differential staining of sister chromatids. Nature 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom. 1981. Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program. Mutation Res. 87:143-188.

14.0 GENERIC PROTOCOL APPROVAL

Gary Roy (SPONSOR REPRESENTATIVE)	7-20-92 DATE
Claire L. Cunningham MA QA AUDITOR	7-22-92 DATE
MA STUDY DIRECTOR	7-21-92 DATE

*15.0 STUDY SPECIFIC PROTOCOL APPROVAL

Nicole E. Cimmerer 10-8-93

MA QA AUDITOR DATE

[signature] 10-8-93

MA STUDY DIRECTOR DATE

*To be completed upon authorization

PROTOCOL APPENDIX A (one 2-page Appendix per Test Material)

TEST AND/OR CONTROL MATERIAL IDENTIFICATION AND HANDLING INFORMATION

This form is designed to specify test material identification, handling and disposition instructions and to provide procedures (if known) in case of accidental exposure to the material. As with all data, this information should be considered confidential and be available only to persons involved with the study or studies using this material.

PRINT OR TYPE THROUGHOUT
USE "NK" IF INFORMATION IS NOT KNOWN; USE "NA" IF NON-APPLICABLE

I. IDENTIFICATION

Test Material ID (Sample No.): FLEXAD (408-93C)
Batch or Lot No.: None CAS No.: 02897-60-1
Physical Description (form): Liquid Color: Clear
Purity (active ingredient): As received
Density: NK Flash Pt: 122°C (closed cup)
Vapor Pressure: NA pH: NK
Solubility: Soluble in acetone
Other: Will react with water
Specific gravity = 0.978

II. STORAGE INFORMATION

Material should be stored in:
Temperature-monitored room 65°-85°F: Yes
Refrigerator: No Freezer: No
Other storage conditions (specify): Cool dry place; protect from moisture

III. STABILITY

Length of time material is stable under conditions described above:
Assumed stable for duration of the study
Expiration Date: NK Indicated on Label: Yes; X No.

Preferred Vehicle: ~~Use N-23 for this study~~ Acetone as per Sponsor PTC 10-6-93

Stability in Vehicle: Unstable, should be tested soon after mixing with N-23

Analysis of neat material or material in vehicle (check if applicable):
1. Will be required
X 2. Will not be required
3. Instructions for analysis will be provided
4. Sample(s) is (are) to be submitted to sponsor for analysis

IV. KNOWN CHEMICAL HAZARDS

Reactivity/Flammability: Reacts with water, moisture
Temperature Range to Avoid: 122°C
Mixtures to Avoid: Water or moist air; oxidizing agents

PROTOCOL APPENDIX A (Cont'd.-Test Material ID (Sample No.): FLEXAD (408-93C)

V. KNOWN OR SUSPECTED TOXICITY HAZARDS

The toxicological properties of this material have not been thoroughly investigated.

Approximate rodent oral LD50: NK mL/Kg _____ Species

Is material probable eye irritant: X Yes; _____ No

Is material probable skin irritant: X Yes; _____ No

Is material probably harmful if inhaled: X Yes; _____ No

Other known toxicity: Mutagenic in Ames "Spot" Test

VI. HANDLING PRECAUTIONS

Use of protective clothing (laboratory coats), latex gloves, safety glasses and dust masks should be routine.

Routine precautions probably adequate: _____ Yes; X No (see below)

Precautions in excess of routine (specify): _____

Emergency Treatment Procedures (if known) in case of accidental exposure are attached: _____ Yes; X No

IN CASE OF EMERGENCY RELATED TO THIS SUBSTANCE, CONTACT:

Brendan J. Dunn (Person) of AlliedSignal Inc. (Company) at 201-455-6077 (Phone #)

VII. DISPOSITION

All materials will be returned to the sponsor (by arrangement) at the address below (IX):

Shipping Instruction (DOT Classification): Nonregulated Research Sample

Materials not in original container (e.g., Archive samples) must be labeled with, at a minimum, sponsor's test material ID (Sample No.). Smaller containers (e.g., <500 mL) should have caps sealed with tape, and should be shipped inside an unbreakable secondary container (e.g., polyethylene bag) within the shipping carton.

VIII. SIGNATURE

Sponsor Signature: Brendan J. Dunn Date: 9-30-93

Brendan J. Dunn, M.S.

Phone Number: (202) 455-6077

IX. SPONSOR NAME AND ADDRESS:

Department of Toxicology
AlliedSignal, Inc.
101 Columbia Road
Morristown, N.J. 07962-1139

s:\toxdoc\bjue\9-28-93a

PROTOCOL AMENDMENT I

SPONSOR:	**ALLIED-SIGNAL INC.**
TEST ARTICLE I.D.:	**FLEXAD (408-93C)**
MA STUDY NO:	**TD894.392006 (Protocol No.: SPGT392006)**
SPONSOR PROJECT NO.:	**93051/TOX-062A**
PROTOCOL TITLE:	***IN VITRO* MAMMALIAN CYTOGETETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES: CONFIRMATORY ASSAY**

---

1. **LOCATION:** Page 2, § 7.0

   AMENDMENT: The second sentence should read: "In the nonactivated test system, exposure will be continuous until harvest;..."

   REASON FOR THE AMENDMENT: To be consistent with section 7.7, second paragraph, for exposure times in the nonactivated assay.

2. **LOCATION:** Protocol Appendix A, § I; page 1 of 2

   AMENDMENT: The color of test article should read "clear and amber".

   REASON FOR THE AMENDMENT: Sponsor request.

APPROVALS:

STUDY DIRECTOR	3-18-94 DATE
SPONSOR REPRESENTATIVE	4-7-94 DATE
Claire L Courtemanche QUALITY ASSURANCE	3-18 94 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

*MA Study Number: TD894.392006

IN VITRO MAMMALIAN CYTOGENETIC TEST USING HUMAN PERIPHERAL LYMPHOCYTES: CONFIRMATORY ASSAY

APPROVED MKR 10-11-95

1.0 PURPOSE

The purpose of this study is to test the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral lymphocytes. This study will be performed to confirm the findings of an earlier cytogenetic test with protocol SPGT392005.

2.0 SPONSOR

2.1 Name: ALLIED-SIGNAL INC.

2.2 Address: Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

2.3 Representative: Gary Roy, M.B.A.

* 2.4 Sponsor Protocol/Project No.: 93051/TOX-062A

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

* 3.1 Sponsor Test Article ID/Sample No.: FLEXAD (408-93C)

3.2 Controls:

Solvent:	Solvent vehicle
Negative:	Untreated cells
Positive:	Mitomycin C and cyclophosphamide

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.

*To be completed upon authorization

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Genetic and Cellular Toxicology Division Microbiological Associates, Inc.
4.2	Address:	9900 Blackwell Road Rockville, MD 20850
4.3	Study Director:	Donald L. Putman, Ph.D.

* 5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 10-12-93

5.2 Proposed Experimental Completion Date: 2-11-94

5.3 Proposed Audited Draft Report Date: 2-13-94

6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from a single healthy adult volunteer without a history of either radiotherapy, viral infections or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing lymphocytes to a minimum of four concentrations of the test article as well as positive and negative controls in duplicate cultures. In the nonactivated test system, exposure will be continuous up to the time of Colcemid treatment; in the S-9 activated system, exposure will be for 4 hours. The dividing cells will be harvested at a two time points, approximately 20 hours from the initiation of treatment (1.5 times normal cell cycle) and 44 hours from the initiation of treatment (24 hours after the initial harvest). The mutagenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

*To be completed upon authorization

7.1 Dose Levels

For the confirmatory cytogenetics assay, lymphocytes will be exposed to at least four concentrations of test article at one-half dilutions, the highest concentration selected according to the findings of the initial assay (SPGT392005) but not to exceed the limit of solubility or 5 mg/ml for solids or 5 ul/ml for liquids, whichever comes first. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition will also be measured.

7.2 Frequency and Route of Administration

Target cells will be treated for approximately 20 hours or 44 hours in the nonactivated system, depending upon harvest time, and for 4 hours in the S-9 activated system. Treatment will be achieved by incorporation of the test article-solvent mixture into the medium. This technique has demonstrated to be an effective method of exposure of mammalian cells in culture to concentrations of chemical.

7.3 Activation System

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The S-9 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 S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with cofactors to contain 2mM $MgCl_2$, 6 mM KCl, 1mM glucose-6-phosphate, 1mM NADP and 20 ul S-9 per ml serum free medium.

7.4 Controls

7.4.1 Untreated Controls

Untreated cells will be used as the untreated control.

7.4.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. The solvents compatible with this test system include water or medium, dimethylsulfoxide, acetone and ethanol. For solvents other than medium, the final concentration in treatment medium will not exceed 1%.

7.4.3 Positive Controls

Mitomycin C (MMC) will be used at two concentrations within 0.10-0.25 ug/ml as the positive control in the non-activated study. Cyclophosphamide (CP) will be used at two concentrations within 25-100 ug/ml as the positive control in the S-9 activated study. For each positive control chemical, a single concentration, the highest with sufficient scorable metaphase cells, will be selected for microscopic evaluation.

7.5 Preparation of Target Cells

Peripheral blood cells will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 ug streptomycin/ml) containing 1% phytohemagglutinin (PHA) by adding 0.6 ml heparinized blood to a centrifuge tube containing 10 ml complete medium with 1% PHA. The tubes will be incubated upright at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 44-48 hours.

7.6 Identification of Test System

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

7.7 Treatment of Target Cells

All treatments will be carried out in duplicate. About 46 hours after culture initiation, tubes will be refed with 10 ml of complete medium for the nonactivated exposure or 10 ml of S-9 reaction mixture for the activated exposure to which will be added 100 ul of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture will also be included.

In the non-activated study, the cells will be exposed for 20 hours or 44 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Treatment will be continuous with Colcemid (0.1 ug/ml) present for the last two hours.

In the S-9 activated study, the cells will be exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium will be aspirated, the cells washed free of chemical and cultured for an additional 16 or 40 hours, depending upon harvest time.

7.8 Collection of Metaphase Cells

Cells will be collected approximately 20 hours or 44 hours after initiation of treatment. This time is selected to assure analysis of first division metaphase cells after initiation of test article treatment as well as any delayed cells. Two hours prior to harvest, Colcemid will be added to the cultures at a final concentration of 0.1 ug/ml. The cells will be collected by centrifugation, treated with 0.075 M KCl, washed with two changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 4±2°C. To prepare slides, the cells will be collected by centrifugation, resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a wet slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

7.9 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted per culture. The percentage of polyploid or endoreduplicated cells per 100 metaphase cells will be determined for each culture.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in the

untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water is used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented. The number and types of aberrations found will be presented for each treatment group. The percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined will be calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) will also be calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells will be performed using the Fisher's exact test and reported for $p \leq 0.05$ and $p \leq 0.01$. Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage trend test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). Treatment groups significantly elevated above the solvent control group at $p \leq 0.01$ will be noted. A reproducible and significant increase at a single dose level will be considered positive.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data. Data will be presented in tabular form for the relative toxic effects of treatment, the number and types of aberrations observed, the frequency of cells with aberrations and the average aberrations per cell.

Results presented for the chromosome aberration assay will include:

- details of the protocol used for preparation of the microsomal enzymes

- dose levels at which the test article was tested
- dose response relationship, if applicable
- historical control data for negative or solvent controls
- evaluation of results
- Microbiological Associates report and study numbers, and Sponsor protocol/project numbers
- identification of test article by Sponsor test material ID and sample number
- observations of any insolubility, film or precipitate of test material and concentrations at which it occurred
- Good Laboratory Practices Compliance Statement, signed by the Study Director
- dates of Quality Assurance reviews of the study, including at least one "in process" inspection of the definitive assay, and of reports to management
- protocols, protocol appendices, and any amendments (signed by Sponsor, Testing Laboratory QA Unit and Study Director) shall be included as report appendices. Deviations from the protocol or Standard Operating Procedures not addressed by protocol amendment will be listed and their impact on the study noted

An audited draft report will be submitted to Sponsor for review and approval. Upon acceptance, five copies of the final report will be sent to Sponsor.

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates Inc. in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EEC Directive 79/831 Annex V, B.10 Mutagenicity (In Vitro Mammalian Cytogenetic Test), June, 1989; OECD Guideline 473 (Genetic Toxicology: In Vitro Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 798.5375 (In Vitro Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. This confirmatory assay will supplement the initial study conducted under protocol SPGT392005 in order to comply with EEC guidelines.

No less than one in-lab audit will be conducted by the Testing Laboratory's QA Unit.

This study will be performed in compliance with the provisions of the US FDA Good Laboratory Practice Regulations (21 CFR 58), the US EPA GLP Regulations (40 CFR 160 and 40 CFR 792), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice.

This study may be submitted to the US EPA and therefore will be listed on the EPA Master Schedule List.

The Test Article will be returned to the Sponsor at the conclusion of the study. Samples of the Test Article dosing solutions are not required by the Sponsor and will be disposed of in accordance with all local, state and federal laws.

13.0 REFERENCES

Evans, H.J. 1976. Cytological methods for detecting chemical mutagens. In: Chemical Mutagens, Principles and Methods for their Detection, vol. 4. A. Hollaender (ed). Plenum Press, New York, NY.

Perry, P. and Wolff, S. 1974. New Giemsa method for differential staining of sister chromatids. Nature 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom. 1981. Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program. Mutation Res. 87:143-188.

14.0 GENERIC PROTOCOL APPROVAL

Gary Roy (SPONSOR REPRESENTATIVE) — DATE 7-20-92

MA QA AUDITOR — DATE 7-22-92

MA STUDY DIRECTOR — DATE 7-21-92

*15.0 STUDY SPECIFIC PROTOCOL APPROVAL

MA QA AUDITOR — DATE 10-8-93

MA STUDY DIRECTOR — DATE 10-8-93

*To be completed upon authorization

PROTOCOL APPENDIX A (one 2-page Appendix per Test Material)

TEST AND/OR CONTROL MATERIAL IDENTIFICATION AND HANDLING INFORMATION

This form is designed to specify test material identification, handling and disposition instructions and to provide procedures (if known) in case of accidental exposure to the material. As with all data, this information should be considered confidential and be available only to persons involved with the study or studies using this material.

PRINT OR TYPE THROUGHOUT
USE "NK" IF INFORMATION IS NOT KNOWN; USE "NA" IF NON-APPLICABLE

I. IDENTIFICATION

Test Material ID (Sample No.): FLEXAD (408-93C)
Batch or Lot No.: None CAS No.: 02897-60-1
Physical Description (form): Liquid Color: Clear
Purity (active ingredient): As received
Density: NK Flash Pt: 122°C (closed cup)
Vapor Pressure: NA pH: NK
Solubility: Soluble in acetone
Other: Will react with water
Specific gravity = 0.978

II. STORAGE INFORMATION

Material should be stored in:
Temperature-monitored room 65°-85°F: Yes
Refrigerator: No Freezer: No
Other storage conditions (specify): Cool dry place; protect from moisture

III. STABILITY

Length of time material is stable under conditions described above:
Assumed stable for duration of the study
Expiration Date: NK Indicated on Label: Yes; X No.

Preferred Vehicle: ~~Use N-23 for this study~~ Acetone as per Sponsor PTC 10-6-93

Stability in Vehicle: Unstable, should be tested soon after mixing with N-23

Analysis of neat material or material in vehicle (check if applicable):
____1. Will be required
_X_2. Will not be required
____3. Instructions for analysis will be provided
____4. Sample(s) is (are) to be submitted to sponsor for analysis

IV. KNOWN CHEMICAL HAZARDS

Reactivity/Flammability: Reacts with water, moisture
Temperature Range to Avoid: 122°C
Mixtures to Avoid: Water or moist air; oxidizing agents

V. KNOWN OR SUSPECTED TOXICITY HAZARDS

The toxicological properties of this material have not been thoroughly investigated.

Approximate rodent oral LD50: NK mL/Kg Species

Is material probable eye irritant: X Yes; No

Is material probable skin irritant: X Yes; No

Is material probably harmful if inhaled: X Yes; No

Other known toxicity: Mutagenic in Ames "Spot" Test

VI. HANDLING PRECAUTIONS

Use of protective clothing (laboratory coats), latex gloves, safety glasses and dust masks should be routine.

Routine precautions probably adequate: Yes; X No (see below)

Precautions in excess of routine (specify):

Emergency Treatment Procedures (if known) in case of accidental exposure are attached: Yes; X No

IN CASE OF EMERGENCY RELATED TO THIS SUBSTANCE, CONTACT:

Brendan J. Dunn (Person) of AlliedSignal Inc. (Company) at 201-455-6077 (Phone #)

VII. DISPOSITION

All materials will be returned to the sponsor (by arrangement) at the address below (IX):

Shipping Instruction (DOT Classification): Nonregulated Research Sample

Materials not in original container (e.g., Archive samples) must be labeled with, at a minimum, sponsor's test material ID (Sample No.). Smaller containers (e.g., <500 mL) should have caps sealed with tape, and should be shipped inside an unbreakable secondary container (e.g., polyethylene bag) within the shipping carton.

VIII. SIGNATURE

Sponsor Signature: Brendan J. Dunn Date: 9-30-93

Brendan J. Dunn, M.S.

Phone Number: (202) 455-6077

IX. SPONSOR NAME AND ADDRESS:

Department of Toxicology
AlliedSignal, Inc.
101 Columbia Road
Morristown, N.J. 07962-1139

s:\tomdoc\byud\9-28-93a

FINAL REPORT

Study Title

SALMONELLA/ESCHERICHIA COLI MUTAGENICITY ASSAY

Test Article

FLEXAD (408-93C)

Sponsor Protocol/Project Number

93050/TOX-062A

Authors

Richard H.C. San, Ph.D.
Jamie E. Sly

RECEIVED
93 JAN -3 PM 3:07

Study Completion Date

12/13/94

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

Laboratory Study Number

TD894.502070

Sponsor

Allied-Signal Inc.
Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

TOX Computer Entry No.
TOX-62A-93-3 12/16/94

STATEMENT OF COMPLIANCE

The *Salmonella/Escherichia coli* Mutagenicity Assay of test article **FLEXAD (408-93C)** was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 792 and 40 CFR 160, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

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

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

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

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

12/13/94
Date

QUALITY ASSURANCE STATEMENT

Study Title: SALMONELLA/ESCHERICHIA COLI MUTAGENICITY ASSAY

Study Number: TD894.502070

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

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, 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.

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

INSPECT ON 10/11/93 - 10/11/93, TO STUDY DIR 10/11/93, TO MGMT 10/11/93
PHASES: PROTOCOL REVIEW

INSPECT ON 10/27/93 - 10/27/93, TO STUDY DIR 10/27/93, TO MGMT 10/27/93
PHASES: TREATMENT AND PLATING OF THE CULTURES

INSPECT ON 01/27/94 - 01/28/94, TO STUDY DIR 01/28/94, TO MGMT 01/31/94
PHASES: DRAFT REPORT

INSPECT ON 12/14/94 - 12/14/94, TO STUDY DIR 12/14/94, TO MGMT 12/14/94
PHASES: 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 — Dec 14, 1994

Claire L. Courtemanche
Quality Assurance Unit

Date

SALMONELLA/ESCHERICHIA COLI MUTAGENICITY ASSAY

FINAL REPORT

Sponsor: **Allied-Signal Inc.**
Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

Authorized Representative: **Gary Roy, M.B.A.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **FLEXAD (408-93C)**

Sponsor Protocol/Project No.: **93050/TOX-062A**

MA Study No.: **TD894.502070**

Test Article Description: **clear amber liquid**

Storage Conditions: **room temperature; protected from exposure to light**

Test Article Receipt: **10/01/93**

Study Initiation: **10/11/93**

Study Director: Richard H.C. San, Ph.D. — 12/13/94 Date

Associate Study Director: Valentine O. Wagner, III — 12/13/94 Date

TABLE OF CONTENTS

	Page
Summary	6
Purpose	7
Materials and Methods	7
Results and Discussion	15
Conclusion	16
Data Tables	17
References	45
Appendix I (Historical Control Data)	46
Appendix II (Study Protocol)	48
Appendix III (Information for Japanese Regulatory Agencies)	64

SUMMARY

The test article, FLEXAD (408-93C), was tested in the *Salmonella* and *E. coli* Mutagenicity Assay using tester strains TA98, TA100, TA1535, TA1537, WP2 *uvr*A (pKM101) and WP2 (pKM101) in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in three phases using the preincubation method. The first phase, the dose range-finding study, was used to establish the dose range for the mutagenicity assay. The second and third phases, the mutagenicity and confirmatory assays, were used to evaluate the mutagenicity of the test article.

In the dose range-finding study, the maximum dose tested was 5000 μg per plate. This dose was delivered to the test system as a clear solution in acetone. The results of the dose range-finding study indicate that mutagenic activity and toxicity but no precipitate were observed. The maximum dose that was plated in the mutagenicity assay was 5000 μg per plate.

In the mutagenicity and confirmatory assays, positive responses were observed with tester strains TA100 (3.2 and 4.1-fold, maximum increases), TA1535 (12.4 and 20.5-fold, maximum increases) and WP2 *uvr*A (pKM101) (2.2 and 3.4-fold, maximum increases) in the presence of S9 activation, and with tester strains TA100 (4.2 and 2.5-fold, maximum increases), TA1535 (9.1 and 12.9-fold, maximum increases) and WP2 *uvr*A (pKM101) (3.6 and 2.6-fold, maximum increases) in the absence of S9 activation. No precipitate but toxicity was observed. The evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation* and Dose Range Tested (μg/plate)											
	TA98		TA100		TA1535		TA1537		WP2 *uvr*A pKM101		WP2 pKM101	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
None	-		+(4.2; 2.5)		+(9.1; 12.9)		-		+(3.6; 2.6)		-	
	33	5000	33	5000	33	5000	33	5000	33	5000	33	5000
Rat	-		+(3.2; 4.1)		+(12.4; 20.5)		-		+(2.2; 3.4)		-	
	100	5000	100	5000	100	5000	100	5000	100	5000	100	5000

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

In conclusion, the results indicate that under the conditions of this study, test article **FLEXAD (408-93C)** did cause positive responses in the *Salmonella/Escherichia coli* Mutagenicity 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 at selected loci of WP2 *uvr*A (pKM101) and WP2 (pKM101) in the presence and absence of S9 activation. This test system has been shown to detect a diverse group of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The ability to induce mutation is indicative of a chemical's genotoxic potential.

MATERIALS AND METHODS

Media and Reagent Preparation

Note: All references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System at MA.

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. When the top agar was not used with S9 or sham mix (e.g., in sterility and titer determinations), 25 ml of water were added for each 100 ml of minimal top agar.

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).

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq$-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 microsomal enzyme mixture (S9 mix) was prepared immediately before its use and contained 10% microsomal enzymes, 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.

Test System

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the potential mutagenic activity of a wide range of chemical classes.

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and Levin *et al.* (1982) and *Escherichia coli* tester strains WP2 *uvr*A (pKM101) and WP2 (pKM101). *Salmonella* tester strains in use at Microbiological Associates, Inc. (MA) were received on 11/10/92. directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley. *E. coli* strains in use at Microbiological Associates, Inc. were received on 07/01/87 (WP2 *uvr*A (pKM101)) and 02/19/93 (WP2 (pKM101)) from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

Genotype of the *Salmonella* Strains Used for Mutagen Testing
Figure 1

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537		*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

The tester strains contain, in addition to a mutation in the histidine operon, additional mutations that enhance their sensitivities to some mutagenic compounds. The *rfa* wall mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals, such as those containing large ring systems that would otherwise be excluded by a normal cell wall. A second mutation is a deletion in the *uvr*B gene that results in a deficient DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, tester strains containing this deletion also require the vitamin biotin for growth.

Tester strains TA98, TA100, WP2 *uvr*A (pKM101) and WP2 (pKM101) also contain the pKM101 plasmid (carrying the R-factor) that further increases the sensitivities of these strains to some mutagens. It has been suggested that this plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

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.

The *E. coli* WP2 *uvr*A (pKM101) and WP2 (pKM101) reverse mutation assay system measures reversion from trp^- to trp^+ at a site blocking a step of tryptophan biosynthesis prior to the formation of anthranilic acid (Brusick *et al.*, 1980). Tryptophan reversion occurs 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).

Frozen Permanent Stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing away approximately 1.5 ml aliquots in glass vials. Frozen Permanent Stocks were stored at $\leq$-70°C. Master plates were prepared by streaking each *Salmonella* tester strain from a frozen permanent stock onto minimal medium supplemented with histidine (260 μM), biotin (3 μM), ampicillin (25 μg/ml) for strains containing the R-factor. Master plates for *E. coli* were prepared by streaking onto nutrient bottom agar. Master plates were incubated at 37$\pm$2°C for 24 to 48 hours and stored at 4$\pm$2°C.

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$\pm$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.

On the day of their use in the mutagenicity assay, all tester strain cultures were checked for the correct genotype. The presence of the *rfa* wall mutation was confirmed for *Salmonella* by demonstration of sensitivity to crystal violet. The deletion in the *uvr*B gene was confirmed for all *Salmonella* by demonstration of sensitivity to ultraviolet light. The presence of the pKM101 plasmid was confirmed for TA98, TA100, WP2 *uvr*A (pKM101) and WP2 (pKM101) by demonstration of resistance to ampicillin. All *E. coli* tester strain WP2 *uvr*A (pKM101) cultures must demonstrate the deletion in the *uvr*A gene by exhibiting sensitivity to ultraviolet light. All *E. coli* tester strain WP2 (pKM101) cultures must demonstrate the presence of the *uvr*A gene by exhibiting resistance to ultraviolet light. All *E. coli* tester strain cultures must demonstrate the presence of the (pKM101) plasmid R-factor by exhibiting resistance to ampicillin. Spontaneous reversion frequencies in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

The test system was exposed to the test article via the preincubation modification of the Ames test (Ames *et al.*, 1975; and updated by Maron and Ames, 1983) originally described by Yahagi *et al.* (1977). This methodology has been shown to detect many classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The assay was performed in three phases. The first phase, the dose-range finding study, 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 TA100 and WP2 *uvr*A (pKM101) on selective minimal agar in both the presence and absence of Aroclor-induced rat liver S9. The second and third phases, the mutagenicity and confirmatory assays, were used to evaluate the potential mutagenicity of the test article. A minimum of five dose levels of test article along with the appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA1537, WP2 *uvr*A (pKM101) and WP2 (pKM101) in the presence and absence of Aroclor-induced rat liver S9. All dose levels of test article, vehicle controls and positive controls were plated in triplicate.

Test and Control Articles

The test article, FLEXAD (408-93C), was received by Microbiological Associates, Inc. on 10/01/93 and was assigned the code number D894. The test article was characterized by the Sponsor as a clear and amber liquid that should be stored in a temperature controlled room at 65-85°F in a cool dry place, protected from moisture. Upon receipt, the test article was described as a clear amber liquid and was stored at room temperature, protected from exposure to light. The vehicle used to deliver FLEXAD (408-93C) to the test system was acetone (CAS# 67-64-1), certified A.C.S., purchased from Fisher Scientific Company. The dosing solutions were not adjusted to compensate for the purity of the test article. Aliquots of dosing solution preparations were not returned to the Sponsor for chemical analysis.

Positive controls plated concurrently with the assay are listed in Figure 2.

Positive Controls
Figure 2

Strain	Activation	Positive Control	Concentration (μg/plate)
TA98	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1537	+	2-aminoanthracene	1.0
	-	9-aminoacridine	75
WP2 *uvr*A (pKM101)	+	2-aminoanthracene	10
	-	methyl methanesulfonate	1000
WP2 (pKM101)	+	sterigmatocystin	100
	-	methyl methanesulfonate	1000

Source and Grade

9-aminoacridine (CAS #134-50-9), Sigma Chemical Co., 98% pure
2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade
methyl methanesulfonate (CAS #66-27-3), Aldrich Chemical Co., 99% pure
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98 % pure
sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade
sterigmatocystin (CAS #10048-13-2), Sigma Chemical Company

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 a volume equal to that used in the assay. To determine the sterility of the S-9 or sham mixes, a 0.5 ml aliquot of S-9 or sham mix was plated on selective agar.

Plating and Scoring Procedures

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 Microbial Mutagenesis Standard Operating Procedures.

The test article dilutions were prepared immediately before use. A 500 µl aliquot of S9 or sham mix was added to 13 X 100 mm glass culture tubes pre-heated to 37±2°C. To these tubes were added 100 µl of appropriate tester strain and either 50 µl of vehicle or test article. When plating the positive controls, the volume of test article was replaced by an appropriate aliquot of positive control. After vortexing, all mixtures were incubated without shaking for 60±2 minutes at 37±2°C. Following the preincubation, 2.0 ml of selective top agar was added to each tube, vortexed and overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates not counted immediately following the incubation period were stored at 4±2°C.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. This toxicity and precipitate were scored relative to the vehicle control plate, using the criteria and codes that appear in Figure 3. Revertant colonies for a given tester strain and activation condition, except for the positive controls, were counted either entirely by automated colony counter or entirely by hand unless 1) the assay is the dose range finding assay, 2) the plate meets the criteria for toxicity defined in the protocol or 3) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually except as noted above.

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. The results of these calculations are presented on the individual strain data forms.

Criteria for a Valid Test and Evaluation of Results

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*), 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 *E. coli* tester strain WP2 *uvr*A (pKM101) cultures must demonstrate the deletion in the *uvr*A gene by exhibiting sensitivity to ultraviolet light. All *E. coli* tester strain WP2 (pKM101) cultures must demonstrate the presence of the *uvr*A gene by exhibiting resistance to ultraviolet light. All *E. coli* tester strain cultures must demonstrate the presence of the (pKM101) plasmid R-factor by exhibiting resistance to ampicillin. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle control as shown below:

TA98	10 - 50
TA100	80 - 240
TA1535	5 - 45
TA1537	3 - 21
WP2 *uvr*A (pKM101)	150 - 380

WP2 (pKM101) 10 - 160

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 three-fold increase in the number of revertants over the mean value of the respective vehicle control with the exception of tester strain WP2 (pKM101) in the presence of S9 activation in which a two-fold increase is required. 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.

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 and TA1537 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 vehicle control value. Data sets for strains TA98, TA100, WP2 *uvr*A (pKM101) and WP2 (pKM101) 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 vehicle control value.

Archives

All experimental records (raw data and appropriate reports) of the study are maintained in the Microbiological Associates, Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850. The Director of the Quality Assurance Unit is responsible for maintaining the archives.

Bacterial Background Lawn Evaluation Code
Figure 3

The condition of the background bacterial lawn is evaluated, first macroscopically and then microscopically (using a dissecting microscope). Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn. The evaluation is recorded using the code described 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 an 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 and an 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 and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	Distinguished by a complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
SP	Slight Precipitate	Distinguished by noticeable precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, normally requiring the plate to be hand counted unless 1) the plate meets the criteria for toxicity defined in the protocol or 2) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count.
HP	Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the normally required hand count difficult unless 1) the plate meets the criteria for toxicity defined in the protocol or 2) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count.

Thus, 3MP would indicate a plate observed to have a moderately reduced background lawn with a marked amount of precipitate that required the plate to be counted manually.

RESULTS AND DISCUSSION

Dose Range-Finding Study

The results of the dose range-finding study are presented in Tables 1 and 2. The maximum dose tested was 5000 μg per plate. This dose was delivered to the test system as a clear solution in acetone using a plating aliquot of 50 μl.

The results of the dose range-finding study indicate that mutagenic activity and toxicity but no precipitate were observed. The maximum dose that was plated in the mutagenicity assay was 5000 μg per plate.

Mutagenicity and Confirmatory Assays

The results of the mutagenicity assay are presented in Tables 3 through 26 and summarized in Tables 27 and 28. These data were generated in Experiments TD894-B1, TD894-B2, TD894-B3, TD894-B4, TD894-B5, TD894-B7. No precipitate but toxicity was observed.

In Experiment TD894-B1, the mutagencitiy assay, positive responses were observed with tester strains TA100 (3.2-fold, maximum increase), TA1535 (12.4-fold, maximum increase) and WP2 *uvr*A (pKM101) (2.2-fold, maximum increase) in the presence of S9 activation and with tester strains TA100 (4.2-fold, maximum increase), TA1535 (9.1-fold, maximum increase) and WP2 *uvr*A (pKM101) (3.6-fold, maximum increase) in the absence of S9 activation. Due to unacceptable vehicle controls, tester strain WP2 (pKM101) in the presence and absence of S9 activation was not evaluated but retested in Experiment TD894-B3.

In Experiment TD894-B2, the confirmatory assay, positive responses were observed with tester strains TA100 (4.1-fold, maximum increase), TA1535 (20.5-fold, maximum increase) and WP2 *uvr*A (pKM101) (3.4-fold, maximum increase) in the presence of S9 activation and with tester strains TA100 (2.5-fold, maximum increase), TA1535 (12.9-fold, maximum increase) and WP2 *uvr*A (pKM101) (2.6-fold, maximum increase) in the absence of S9 activation. Due to the presence of numerous intermediate-sized (non-revertant) colonies, which interfered with the counting of the revertant colonies, tester strain TA98 in the presence of S9 activation was not evaluated but was retested in Experiment TD894-B6. Due to an unacceptable positive control value, tester strain WP2 (pKM101) in the presence of S9 activation was not evaluated but retested in Experiment TD894-B5.

In Experiment TD894-B3, no positive response was observed with tester strain WP2 (pKM101) in the absence of S9 activation. Due to an unacceptable positive control value, tester strain WP2 (pKM101) in the presence of S9 activation was not evaluated but retested in Experiment TD894-B4.

In Experiment TD894-B4, no positive response was observed with tester strain WP2 (pKM101) in the presence of S9 activation.

In Experiment TD894-B5, no positive response was observed with tester strain WP2 (pKM101) in the presence of S9 activation.

In Experiment TD894-B6, due to an unacceptable vehicle control value, tester strain TA98 in the presence of S9 activation was not evaluated but retested in Experiment TD894-B7.

In Experiment TD894-B7, no positive response was observed with tester strain TA98 in the presence of S9 activation.

All criteria for a valid study were met as described in the protocol.

CONCLUSION

The results of the *Salmonella/Escherichia coli* Mutagenicity Assay indicate that under the conditions of this study, **Allied-Signal Inc.'s** test article **FLEXAD (408-93C)** did cause positive responses with tester strains TA100 (3.2 and 4.1-fold, maximum increases), TA1535 (12.4 and 20.5-fold, maximum increases) and WP2 *uvr*A (pKM101) (2.2 and 3.4-fold, maximum increases) in the presence of Aroclor-induced rat liver S9, and with tester strains TA100 (4.2 and 2.5-fold, maximum increases), TA1535 (9.1 and 12.9-fold, maximum increases) and WP2 *uvr*A (pKM101) (3.6 and 2.6-fold, maximum increases) in the absence of Aroclor-induced rat liver S9.

Salmonella Mutagenicity Assay

Dose Range-Finding Study

Table 1

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070
Experiment No. : A1
Date Plated : 10/13/93
Counted by : machine
Vehicle : acetone
Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA100 With S9 Activation Revertants per plate	TA100 With S9 Activation Background Code[a]	TA100 Without Activation Revertants per plate	TA100 Without Activation Background Code[a]
Vehicle	181	1	188	1
6.7	202	1	185	1
10	209	1	161	1
33	211	1	194	1
67	213	1	213	1
100	234	1	201	1
333	218	1	362	1
667	251	1	478	1
1000	289	1	556	1
3333	728	2	0[b]	5
5000	1219	2	0[b]	5

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

E. coli Mutagenicity Assay

Dose Range-Finding Study

Table 2

Test Article Id	FLEXAD (408-93C)
Study Number	TD894.502070
Experiment No.	A1
Date Plated	10/13/93
Counted by	machine
Vehicle	acetone
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	274	1	203	1
6.7	288	1	258	1
10	303	1	260	1
33	363	1	247	1
67	404	1	265	1
100	390	1	261	1
333	435	1	433	1
667	392	1	565	1
1000	364	1	634	1
3333	974	1	575	3
5000	927	1	31[b]	4

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plate was hand counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070 Experiment No : B1
Strain : TA98 Cells Seeded : 0.7 X 10^8
Liver Microsomes : None Date Plated : 10/27/93
Vehicle : acetone
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	22	1		
	02	24	1		
	03	23	1	23	1
33	01	32	1		
	02	23	1		
	03	32	1	29	5
100	01	12	1		
	02	22	1		
	03	15	1	16	5
333	01	22	1		
	02	25	1		
	03	25	1	24	2
1000	01	25	1		
	02	16	1		
	03	14	1	18	6
3333	01	20	1		
	02	13	2		
	03	9	2	14	6
5000	01	0	5		
	02	0	5		
	03	0	4	0	0
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	488	1		
	02	426	1		
	03	243	1	386	127

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 4

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA98	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 10/27/93
Vehicle	: acetone		
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	27	1		
	02	30	1		
	03	20	1	26	5
100	01	30	1		
	02	23	1		
	03	21	1	25	5
333	01	23	1		
	02	39	1		
	03	27	1	30	8
1000	01	49	1		
	02	27	1		
	03	34	1	37	11
2000	01	33	1		
	02	37	1		
	03	40	1	37	4
3333	01	37	1		
	02	33	1		
	03	31	1	34	3
5000	01	14	3		
	02	0	4		
	03	0	4	5	8
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	1554	1		
	02	1674	1		
	03	1590	1	1606	62

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 5

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA100	Cells Seeded	: 0.5 X 10^8
Liver Microsomes	: None	Date Plated	: 10/27/93
Vehicle	: acetone		
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	120	1		
	02	127	1		
	03	151	1	133	16
33	01	151	1		
	02	157	1		
	03	158	1	155	4
100	01	191	1		
	02	184	1		
	03	165	1	180	13
333	01	316	1		
	02	277	1		
	03	257	1	283	30
1000	01	553	1		
	02	576	1		
	03	558	1	562	12
3333	01[b]	0	4		
	02[b]	0	4		
	03	651	2	217	376
5000[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control sodium azide 1.0 µg per plate					
	01	639	1		
	02	577	1		
	03	586	1	601	34

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 6

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070
Strain : TA100
Liver Microsomes : Rat liver S9
Vehicle : acetone
Plating Aliquot : 50 µl

Experiment No : B1
Cells Seeded : 0.5 X 10^8
Date Plated : 10/27/93
Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	174	1		
	02	180	1		
	03	144	1	166	19
100	01	159	1		
	02	190	1		
	03	145	1	165	23
333	01	170	1		
	02	191	1		
	03	176	1	179	11
1000	01	260	1		
	02	257	1		
	03	267	1	261	5
2000	01	457	1		
	02	484	1		
	03	421	1	454	32
3333	01	427	2		
	02	571	2		
	03	602	2	533	93
5000[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	1670	1		
	02	1920	1		
	03	2229	1	1940	280

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 7

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA1535	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: None	Date Plated	: 10/27/93
Vehicle	: acetone		
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	18	1		
	02	14	1		
	03	16	1	16	2
33	01	22	1		
	02	20	1		
	03	17	1	20	3
100	01	32	1		
	02	33	1		
	03	27	1	31	3
333	01	90	1		
	02[b]	84	1		
	03[b]	80	1	85	5
1000[b]	01	152	1		
	02	142	1		
	03	140	1	145	6
3333	01[b]	90	3		
	02	34	3		
	03[b]	175	2	100	71
5000	01	0	4		
	02	0	4		
	03[b]	127	2	42	73
Positive Control sodium azide 1.0 µg per plate[b]					
	01	403	1		
	02	406	1		
	03	432	1	414	16

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were machine counted

Salmonella Mutagenicity Assay

Table 8

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA1535	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 10/27/93
Vehicle	: acetone		
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	16	1		
	02	17	1		
	03	15	1	16	1
100	01	19	1		
	02	18	1		
	03	16	1	18	2
333	01	22	1		
	02	22	1		
	03	17	1	20	3
1000	01	56	1		
	02[b]	68	1		
	03[b]	58	1	61	6
2000[b]	01	200	1		
	02	197	1		
	03	200	1	199	2
3333[b]	01	67	3		
	02	196	2		
	03	208	2	157	78
5000	01	0	5		
	02	0	5		
	03	0	4	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	107	1		
	02	106	1		
	03	101	1	105	3

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were machine counted

Salmonella Mutagenicity Assay

Table 9

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA1537	Cells Seeded	: 0.4 X 10^8
Liver Microsomes	: None	Date Plated	: 10/27/93
Vehicle	: acetone		
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	6	1		
	02	5	1		
	03	3	1	5	2
33	01	5	1		
	02	4	1		
	03	11	1	7	4
100	01	4	1		
	02	7	1		
	03	2	1	4	3
333	01	6	1		
	02	3	1		
	03	9	1	6	3
1000	01	8	1		
	02	5	1		
	03	7	1	7	2
3333	01	7	1		
	02	5	2		
	03	6	1	6	1
5000	01	0	5		
	02	0	4		
	03	0	4	0	0
Positive Control 9-aminoacridine 75 µg per plate[b]					
	01	717	1		
	02	450	1		
	03	483	1	550	146

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 10

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: TA1537	Cells Seeded	: 0.4 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 10/27/93
Vehicle	: acetone		
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	6	1		
	02	9	1		
	03	2	1	6	4
100	01	7	1		
	02	0	3		
	03	6	1	4	4
333	01	7	1		
	02	7	1		
	03	6	1	7	1
1000	01	6	1		
	02	5	1		
	03	8	1	6	2
2000	01	4	1		
	02	5	1		
	03	10	1	6	3
3333	01	6	1		
	02	4	3		
	03	8	1	6	2
5000	01	13	1		
	02	0	4		
	03	0	4	4	8
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	252	1		
	02	330	1		
	03	278	1	287	40

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

E. coli Mutagenicity Assay

Table 11

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: WP2 *uvrA* (pKM101)	Cells Seeded	: 2.1 X 10^8
Liver Microsomes	: None	Date Plated	: 10/27/93
Vehicle	: acetone		
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	278	1		
	03	258	1	259	18
33	01	173	1		
	02	281	1		
	03	259	1	238	57
100	01	248	1		
	02	302	1		
	03	311	1	287	34
333	01	370	1		
	02	389	1		
	03	331	1	363	30
1000	01	673	1		
	02	666	1		
	03	661	1	667	6
3333	01	698	2		
	02	981	1		
	03	1087	1	922	201
5000[b]	01	0	4		
	02	0	4		
	03	0	4	0	0
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	2214	1		
	02	1920	1		
	03	1652	1	1929	281

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

E. coli Mutagenicity Assay

Table 12

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B1
Strain	: WP2 *uvrA* (pKM101)	Cells Seeded	: 2.1 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 10/27/93
Vehicle	: acetone		
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	309	1		
	02	282	1		
	03	298	1	296	14
100	01	293	1		
	02	338	1		
	03	310	1	314	23
333	01	352	1		
	02	357	1		
	03	369	1	359	9
1000	01	443	1		
	02	412	1		
	03	423	1	426	16
2000	01	536	1		
	02	560	1		
	03	541	1	546	13
3333	01	630	1		
	02	616	1		
	03	719	1	655	56
5000	01	513	3		
	02	452	3		
	03	550	2	505	49
Positive Control 2-aminoanthracene 10 µg per plate					
	01	2046	1		
	02	1795	1		
	03	1863	1	1901	130

[a]Background bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

Salmonella Mutagenicity Assay

Table 13

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA98	Cells Seeded	: 0.8 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	18	1		
	02	19	1		
	03	25	1	21	4
33	01	21	1		
	02	23	1		
	03	21	1	22	1
100	01	27	1		
	02	18	1		
	03	19	1	21	5
333	01	22	1		
	02	32	1		
	03	31	1	28	6
1000	01	21	1		
	02	27	1		
	03	34	1	27	7
3333	01	22	1		
	02	0	4		
	03	0	4	7	13
5000	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	398	1		
	02	1461	1		
	03	1547	1	1135	640

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 14

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA100	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	NC			
	02	147	1		
	03	179	1	163	23
33	01	148	1		
	02	166	1		
	03	156	1	157	9
100	01	179	1		
	02	200	1		
	03	NC		190	15
333	01	287	1		
	02	306	1		
	03	285	1	293	12
1000	01	412	1		
	02	321	1		
	03	479	1	404	79
3333[b]	01	0	4		
	02	0	5		
	03	0	5	0	0
5000[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control sodium azide 1.0 µg per plate					
	01	531	1		
	02	588	1		
	03	634	1	584	52

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

NC=No count due to procedural error in which plate did not receive an aliquot of tester strain

Salmonella Mutagenicity Assay

Table 15

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070 Experiment No : B2
Strain : TA100 Cells Seeded : 0.7 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 11/16/93
Vehicle : acetone
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	160	1		
	02	142	1		
	03	174	1	159	16
100	01	180	1		
	02	167	1		
	03	165	1	171	8
333	01	186	1		
	02	225	1		
	03	159	1	190	33
1000	01	240	1		
	02	249	1		
	03	270	1	253	15
2000	01	451	1		
	02	340	1		
	03	353	1	381	61
3333	01	714	1		
	02	612	1		
	03	641	1	656	53
5000	01	0[b]	4		
	02	0[b]	4		
	03	892	1	297	515
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	1350	1		
	02	1348	1		
	03	1447	1	1382	57

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 16

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA1535	Cells Seeded	: 0.8 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	9	1		
	02	14	1		
	03	5	1	9	5
33	01	20	1		
	02	16	1		
	03	7	1	14	7
100	01	20	1		
	02	24	1		
	03	26	1	23	3
333	01	56	1		
	02	73	1		
	03	45	1	58	14
1000	01	123	1		
	02	120	1		
	03	105	1	116	10
3333	01	0[b]	5		
	02	0[b]	5		
	03	127	1	42	73
5000[b]	01	0	4		
	02	0	5		
	03	0	5	0	0
Positive Control sodium azide 1.0 µg per plate					
	01	600	1		
	02	561	1		
	03	602	1	588	23

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 17

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA1535	Cells Seeded	: 0.8 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 11/16/93
Vehicle	: acetone		
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	15	1		
	02	25	1		
	03	19	1	20	5
100	01	14	1		
	02	15	1		
	03	25	1	18	6
333	01	23	1		
	02	28	1		
	03	22	1	24	3
1000	01	60	1		
	02	57	1		
	03	63	1	60	3
2000	01	176	1		
	02	180	1		
	03	179	1	178	2
3333	01	337	1		
	02	328	1		
	03	131	1	265	116
5000	01	500	1		
	02	389	2		
	03	340	1	410	82
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	138	1		
	02	144	1		
	03	164	1	149	14

[a]Background bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

Salmonella Mutagenicity Assay

Table 18

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA1537	Cells Seeded	: 0.3 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	9	1		
	02	6	1		
	03	6	1	7	2
33	01	6	1		
	02	9	1		
	03	4	1	6	3
100	01	6	1		
	02	8	1		
	03	5	1	6	2
333	01	8	1		
	02	7	1		
	03	NC		8	1
1000	01	6	1		
	02	7	1		
	03	6	1	6	1
3333	01	0	3		
	02	0	5		
	03	0	3	0	0
5000	01	0	5		
	02	0	4		
	03	0	5	0	0
Positive Control 9-aminoacridine 75 µg per plate[b]					
	01	790	1		
	02	729	1		
	03	724	1	748	37

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

NC=No count due to procedural error in which plate did not receive an aliquot of tester strain

Salmonella Mutagenicity Assay

Table 19

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: TA1537	Cells Seeded	: 0.3 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 11/16/93
Vehicle	: acetone		
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	7	1		
	02	9	1		
	03	4	1	7	3
100	01	4	1		
	02	7	1		
	03	3	1	5	2
333	01	17	1		
	02	4	1		
	03	4	1	8	8
1000	01	10	1		
	02	10	1		
	03	2	1	7	5
2000	01	7	1		
	02	3	1		
	03	9	1	6	3
3333	01	2	2		
	02	6	2		
	03	1	2	3	3
5000	01	5	2		
	02	7	2		
	03	5	2	6	1
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	126	1		
	02	412	1		
	03	827	1	455	352

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

E. coli Mutagenicity Assay

Table 20

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: WP2 *uvrA* (pKM101)	Cells Seeded	: 1.9×10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	262	1		
	02	245	1		
	03	245	1	251	10
33	01	214	1		
	02	204	1		
	03	214	1	211	6
100	01	275	1		
	02	245	1		
	03	219	1	246	28
333	01	336	1		
	02	362	1		
	03	322	1	340	20
1000	01	497	1		
	02	512	1		
	03	540	1	516	22
3333	01	741	2		
	02	712	2		
	03	498	2	650	133
5000[b]	01	0	4		
	02	0	4		
	03	0	4	0	0
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	2465	1		
	02	2423	1		
	03	2472	1	2453	27

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

E. coli Mutagenicity Assay

Table 21

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070 Experiment No : B2
Strain : WP2 *uvrA* (pKM101) Cells Seeded : 1.9 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 11/16/93
Vehicle : acetone
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	214	1		
	02	303	1		
	03	283	1	267	47
100	01	240	1		
	02	280	1		
	03	304	1	275	32
333	01	313	1		
	02	292	1		
	03	279	1	295	17
1000	01	224	2		
	02	361	1		
	03	359	1	315	79
2000	01	608	1		
	02	465	1		
	03	572	1	548	74
3333	01	814	1		
	02	399	1		
	03	842	1	685	248
5000	01	1075	1		
	02	876	1		
	03	787	1	913	147
Positive Control 2-aminoanthracene 10 µg per plate					
	01	2038	1		
	02	1898	1		
	03	1995	1	1977	72

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

E. coli Mutagenicity Assay

Table 22

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B2
Strain	: WP2 (pKM101)	Cells Seeded	: 1.8 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	NC			
	02	85	1		
	03	92	1	89	5
33	01	77	1		
	02	118	1		
	03	101	1	99	21
100	01	62	1		
	02	73	1		
	03	79	1	71	9
333	01	57	1		
	02	86	1		
	03	91	1	78	18
1000	01	107	1		
	02	100	1		
	03	88	1	98	10
3333[b]	01	0	4		
	02	0	4		
	03	0	4	0	0
5000[b]	01	0	4		
	02	0	4		
	03	0	4	0	0
Positive Control methyl methanesulfonate				1000 µg per plate	
	01	1365	1		
	02	1826	1		
	03	1757	1	1649	249

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

NC=No count due to procedural error in which plate did not receive an aliquot of tester strain

E. coli Mutagenicity Assay

Table 23

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B3
Strain	: WP2 (pKM101)	Cells Seeded	: 1.6 X 10^8
Liver Microsomes	: None	Date Plated	: 11/16/93
Vehicle	: acetone		
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	60	1		
	02	55	1		
	03	50	1	55	5
33	01	86	1		
	02	70	1		
	03	55	1	70	16
100	01	64	1		
	02	78	1		
	03	70	1	71	7
333	01	NC			
	02	59	1		
	03	63	1	61	3
1000	01	91	1		
	02	90	1		
	03	90	1	90	1
3333[b]	01	10	3		
	02	0	3		
	03	0	3	3	6
5000[b]	01	5	3		
	02	7	3		
	03	0	4	4	4
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	2249	1		
	02	2045	1		
	03	2087	1	2127	108

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

NC=No count due to procedural error in which plate did not receive an aliquot of tester strain

E. coli Mutagenicity Assay

Table 24

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B4
Strain	: WP2 (pKM101)	Cells Seeded	: 8.2×10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 12/03/93
Vehicle	: acetone		
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	126	1		
	02	130	1		
	03	105	1	120	13
100	01	158	1		
	02	159	1		
	03	138	1	152	12
333	01	132	1		
	02	103	1		
	03	122	1	119	15
1000	01	129	1		
	02	134	1		
	03	NC		132	4
2000	01	108	1		
	02	137	1		
	03	125	1	123	15
3333	01	136	1		
	02	151	1		
	03	124	1	137	14
5000	01	134	1		
	02	124	1		
	03	92	1	117	22
Positive Control sterigmatocystin 100 µg per plate					
	01	257	1		
	02	268	1		
	03	NC		263	8

[a]Background bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
NC=No count due to procedural error in which plates were inadvertently not plated

E. coli Mutagenicity Assay

Table 25

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B5
Strain	: WP2 (pKM101)	Cells Seeded	: 7.3 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 12/03/93
Vehicle	: acetone		
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	131	1		
	02	130	1		
	03	85	1	115	26
100	01	104	1		
	02	144	1		
	03	129	1	126	20
333	01	141	1		
	02	134	1		
	03	134	1	136	4
1000	01	131	1		
	02	133	1		
	03	145	1	136	8
2000	01	89	1		
	02	122	1		
	03	83	1	98	21
3333[b]	01	6	3		
	02	0	3		
	03	27	3	11	14
5000[b]	01	0	4		
	02	0	4		
	03	0	4	0	0
Positive Control sterigmatocystin 100 µg per plate					
	01	253	1		
	02	245	1		
	03	240	1	246	7

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 26

Test Article Id	: FLEXAD (408-93C)		
Study Number	: TD894.502070	Experiment No	: B7
Strain	: TA98	Cells Seeded	: 3.4 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 01/05/94
Vehicle	: acetone		
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	28	1		
	02	31	1		
	03	33	1	31	3
100	01	41	1		
	02	24	1		
	03	25	1	30	10
333	01	31	1		
	02	37	1		
	03	37	1	35	3
1000	01	35	1		
	02	41	1		
	03	29	1	35	6
2000	01	45	1		
	02	43	1		
	03	43	1	44	1
3333	01	13	1		
	02	55	1		
	03	44	1	37	22
5000	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	1195	1		
	02	1168	1		
	03	1233	1	1199	33

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella/E. coli Mutagenicity Assay
Summary of Results

Table 27

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070 Experiment Nos : B1/B3/B4

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)	WP2 (pKM101)
0.0	23 ± 1	133 ± 16	16 ± 2	5 ± 2	259 ± 18	55 ± 5
33	29 ± 5	155 ± 4	20 ± 3	7 ± 4	238 ± 57	70 ± 16
100	16 ± 5	180 ± 13	31 ± 3	4 ± 3	287 ± 34	71 ± 7
333	24 ± 2	283 ± 30	85 ± 5	6 ± 3	363 ± 30	61 ± 3
1000	18 ± 6	562 ± 12	145 ± 6	7 ± 2	667 ± 6	90 ± 1
3333	14 ± 6	217 ± 376	100 ± 71	6 ± 1	922 ± 201	3 ± 6
5000	0 ± 0	0 ± 0	42 ± 73	0 ± 0	0 ± 0	4 ± 4
Pos	386 ± 127	601 ± 34	414 ± 16	550 ± 146	1929 ± 281	2127 ± 108

Liver Microsomes: Rat liver S9

Dose (µg)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)	WP2 (pKM101)
0.0	26 ± 5	166 ± 19	16 ± 1	6 ± 4	296 ± 14	120 ± 13
100	25 ± 5	165 ± 23	18 ± 2	4 ± 4	314 ± 23	152 ± 12
333	30 ± 8	179 ± 11	20 ± 3	7 ± 1	359 ± 9	119 ± 15
1000	37 ± 11	261 ± 5	61 ± 6	6 ± 2	426 ± 16	132 ± 4
2000	37 ± 4	454 ± 32	199 ± 2	6 ± 3	546 ± 13	123 ± 15
3333	34 ± 3	533 ± 93	157 ± 78	6 ± 2	655 ± 56	137 ± 14
5000	5 ± 8	0 ± 0	0 ± 0	4 ± 8	505 ± 49	117 ± 22
Pos	1606 ± 62	1940 ± 280	105 ± 3	287 ± 40	1901 ± 130	263 ± 8

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

Salmonella/E. coli Confirmatory Assay
Summary of Results

Table 28

Test Article Id : FLEXAD (408-93C)
Study Number : TD894.502070 Experiment Nos : B2/B5/B7

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)	WP2 (pKM101)
0.0	21 ± 4	163 ± 23	9 ± 5	7 ± 2	251 ± 10	89 ± 5
33	22 ± 1	157 ± 9	14 ± 7	6 ± 3	211 ± 6	99 ± 21
100	21 ± 5	190 ± 15	23 ± 3	6 ± 2	246 ± 28	71 ± 9
333	28 ± 6	293 ± 12	58 ± 14	8 ± 1	340 ± 20	78 ± 18
1000	27 ± 7	404 ± 79	116 ± 10	6 ± 1	516 ± 22	98 ± 10
3333	7 ± 13	0 ± 0	42 ± 73	0 ± 0	650 ± 133	0 ± 0
5000	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pos	1135 ± 640	584 ± 52	588 ± 23	748 ± 37	2453 ± 27	1649 ± 249

Liver Microsomes: Rat liver S9

Dose (µg)	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)	WP2 (pKM101)
0.0	31 ± 3	159 ± 16	20 ± 5	7 ± 3	267 ± 47	115 ± 26
100	30 ± 10	171 ± 8	18 ± 6	5 ± 2	275 ± 32	126 ± 20
333	35 ± 3	190 ± 33	24 ± 3	8 ± 8	295 ± 17	136 ± 4
1000	35 ± 6	253 ± 15	60 ± 3	7 ± 5	315 ± 79	136 ± 8
2000	44 ± 1	381 ± 61	178 ± 2	6 ± 3	548 ± 74	98 ± 21
3333	37 ± 22	656 ± 53	265 ± 116	3 ± 3	685 ± 248	11 ± 14
5000	0 ± 0	297 ± 515	410 ± 82	6 ± 1	913 ± 147	0 ± 0
Pos	1199 ± 33	1382 ± 57	149 ± 14	455 ± 352	1977 ± 72	246 ± 7

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

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., V.F. Simmon, H.S. Rosenkranz, V.A. Ray, and R.S. Stafford (1980) An Evaluation of the *Escherichia coli* WP_2 and WP_2 *uvr*A 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., Hollstein, M.C., Christman, M.F., Schwiers, E.A. and Ames, B.N. (1982). A new *Salmonella* tester strain (TA102) with A:T base pairs at the site of mutation detects oxidative mutagens. Proc. Natl. Acad. Sci. USA 79:7445-7449.

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

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

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

Yahagi, M., Y. Nagao, T. Seino, T. Sugimura and M. Okada (1977) Mutagenicities of N-nitrosamines on *Salmonella*, Mutation Research 48:121-130.

APPENDIX I

Historical Control Data

Historical Vehicle and Positive Control Values
Preincubation Method

FY1990 - FY1992

S9 Activation	Parameter	TA98		TA100		TA1535		TA1537		TA1538		WP2 *uvrA*[a] (pKM101)		WP2[a] (pKM101)	
		Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos
None	Mean	20	237	131	652	9	416	7	630	16	239	238	2146	54	1795
	SD	7	76	33	175	3	161	4	371	4	61	51	503	15	506
	Minimum	8	90	68	327	2	69	0	92	8	120	134	1258	30	905
	Maximum	49	614	218	1568	20	1346	22	2922	30	438	316	2867	82	2892
Rat	Mean	24	1232	167	1140	11	112	8	191	12	1268	282	1713	65	262
	SD	8	448	39	384	4	70	4	314	4	487	49	475	17	123
	Minimum	3	72	53	375	2	39	1	34	4	64	199	923	38	113
	Maximum	48	2506	251	2456	37	498	24	2697	33	2567	380	2468	89	453

Veh = Vehicle Control (including but not limited to deionized water, dimethylsulfoxide, ethanol and acetone)
Pos = Positive Control
a = Data collected during FY1993 when these strains were introduced into the experimental design

APPENDIX II

Study Protocol

PROTOCOL AMENDMENT I

SPONSOR: **Allied-Signal Inc.**

TEST ARTICLE I.D.: **FLEXAD (408-93C)**

MA STUDY NO: **TD894.502070**

SPONSOR PROJECT NO.: **93050/TOX-062A**

PROTOCOL TITLE: ***Salmonella/Escherichia coli* Mutagenicity Assay**

---

1. **LOCATION:** Page 8, § 8.4, Positive Control Values

AMENDMENT: Paragraph should read:

Each mean positive control value must exhibit at least a three-fold increase over the respective mean vehicle control value for each tester strain with the exception of tester strain WP2 (pKM101) in the presence of S9 activation in which a two-fold increase will be required.

REASON FOR THE AMENDMENT: Tester strain WP2 (pKM101) lacks the *uvr*A deletion, making it DNA repair proficient. Consequently, it has the ability to efficiently repair the DNA damage induced by sterigmatocystin and other positive control articles. In this study, sterigmatocystin (STM) preincubated at 37°C for 60 minutes at 100 µg/plate routinely yields only 300 to 400 revertants/plate, making it difficult to consistently demonstrate a three-fold increase over the vehicle control value. The inclusion of several additional concentrations of STM (50 to 200 µg/plate) did not show an appreciable difference in the response. In addition, studies on the effect of preincubation duration (20 to 60 minutes) indicate that, with a 60-minute preincubation, the response of the bacteria diminishes to a two to three-fold increase over the vehicle control value. In light of these limitations, the mean positive control value for tester strain WP2 (pKM101) in the presence of S9 activation must exhibit at least a two-fold increase over its respective mean vehicle control value.

APPROVALS:

STUDY DIRECTOR	12/20/93 DATE
SPONSOR REPRESENTATIVE	1-5-94 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

PROTOCOL AMENDMENT II

SPONSOR: **Allied-Signal Inc.**

TEST ARTICLE I.D.: **FLEXAD (408-93C)**

MA STUDY NO: **TD894.502070**

SPONSOR PROJECT NO.: **93050/TOX-062A**

PROTOCOL TITLE: ***Salmonella/Escherichia coli* Mutagenicity Assay**

---

1. **LOCATION:** Protocol Appendix A, Page 1, § I, Physical Description

AMENDMENT: Color should read:

Clear and amber

REASON FOR THE AMENDMENT: To resolve physical description of test article. Upon receipt, MA described TA as clear amber liquid. On the protocol Appendix A, Sponsor instructed MA to change description from clear liquid to clear and amber liquid.

APPROVALS:

STUDY DIRECTOR	1/31/94 DATE
Claire L Courtemanche QUALITY ASSURANCE	1/31/94 DATE
SPONSOR REPRESENTATIVE	2.2.94 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

Received by RA/QA 10-11-93

MA Study Number: TD894.502070

APPROVED MKS 10-11-93

SALMONELLA/ESCHERICHIA COLI MUTAGENICITY 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* tester strains WP2 (pKM101) and WP2 *uvr*A (pKM101) in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: **ALLIED-SIGNAL INC.**

2.2 Address: Department of Toxicology
101 Columbia Road
Morristown, NJ 07962-1139

2.3 Representative: Gary Roy, M.B.A.

*2.4 Sponsor Protocol/Project No.: 93050/TOX-062A

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

*3.1 Sponsor Test Material I.D. (Sample No.): FLEXAD (408-93C)

3.2 Controls: Negative: Test article vehicle

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

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. See attached Protocol Appendix A for safety and handling information.

*To be completed upon authorization.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic and Cellular Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

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

*5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 10/13/93

5.2 Proposed Experimental Completion Date: 11/26/93

5.3 Proposed Report Date: 12/3/93

*To be completed upon authorization.

6.0 TEST SYSTEM

6.1 *S. typhimurium* Tester Strains

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537		*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two 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 second mutation is a deletion in the *uvr*B gene resulting 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 *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

6.2 *E. coli* Tester Strains

The *E. coli* reverse mutation assay system measures reversion from trp^- to trp^+ at a site blocking a step of tryptophan biosynthesis prior to the formation of anthranilic acid. Both tester strains WP2 (pKM101) and WP2 *uvr*A (pKM101) have an AT base pair at the critical mutation site within the *trpE* gene and carry the pKM101 plasmid to enhance error-prone repair (Wilcox *et al.*, 1990). Tester strain WP2 *uvr*A (pKM101) is excision repair deficient. However, tester strain WP2 (pKM101) is excision repair proficient and thus will detect cross-linking agents. 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).

E. coli tester strains WP2 (pKM101) and WP2 *uvr*A (pKM101) were 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 vehicle and positive controls with tester strains TA98, TA100, TA1535, TA1537, WP2 (pKM101) and WP2 *uvr*A (pKM101) with and without S9 activation. All dose levels of test article, vehicle 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 TA100 and WP2 *uvr*A (pKM101) to vehicle and to at least eight concentrations of test article, one plate per dose level, in 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 both microscopically and macroscopically following the incubation period. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity. In the event that the test article cannot be dissolved 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.

7.3 Frequency and Route of Administration

The test system will be exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi *et al.* (1977). 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 execessive 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	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1537	+	2-aminoanthracene	1.0
	-	9-aminoacridine	75
WP2 (pKM101)	+	sterigmatocystin	100
	-	methyl methanesulfonate	1000
WP2 *uvr*A (pKM101)	+	2-aminoanthracene	10
	-	methyl methanesulfonate	1000

7.4.2 Vehicle Controls

Appropriate vehicle controls will be plated for each tester strain with and without S9 activation.

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 will be added to pre-heated 13 x 100 mm glass culture tubes. To these tubes will be added 100 µl of tester strain and 50 µl of vehicle, test article dilution or positive control. After vortexing, the mixture will be allowed to incubate for 60±2 minutes at 37±2°C. Two milliliters of selective top agar will then be added to each tube and the mixture will be 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. When necessary to achieve the target concentration, aliquots of other than 50 µl of test article/vehicle/positive control will be plated. 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 vehicle 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 by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

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

The presence of the *uvr*A mutation in WP2 *uvr*A (pKM101) will be confirmed by demonstrating sensitivity to ultraviolet light. The absence of the *uvr*A mutation in WP2 (pKM101) will be confirmed by demonstrating resistance to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for both tester strains 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 *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.

To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain WP2 *uvr*A (pKM101) cultures must exhibit sensitivity to ultraviolet light. To demonstrate the absence of the *uvr*A mutation, all *E. coli* tester strain WP2 (pKM101) cultures must exhibit resistance to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, both *E. coli* tester strain cultures must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. 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 (pKM101), 10 - 160; WP2 *uvr*A (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 $0.3x10^9$ 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 vehicle control value 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 vehicle 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 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 three times the mean vehicle control value.

9.2 Strains TA98, TA100, WP2 (pKM101) and WP2 *uvr*A (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 vehicle control value.

10.0 REPORT

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

- bacterial tester strain description

- test conditions, including dose levels and rationale for selection, number of plates per test point, toxicity, media, type and composition of metabolic activation system, treatment procedures, positive and negative controls.
- individual plate counts
- mean and standard deviation of revertant colonies per plate
- dose response relationship, if applicable
- evaluation of results
- historical control values
- Microbiological Associates report and study numbers, and Sponsor protocol/project numbers
- identification of test article by Sponsor test material ID and sample number
- observations of any insolubility, film or precipitate of test material and concentrations at which it occurred
- Good Laboratory Practices Compliance Statement, signed by the Study Director
- dates of Quality Assurance reviews of the study, including at least one "in process" inspection of the definitive assay, and of reports to management
- protocols, protocol appendices, and any amendments (signed by Sponsor and Study Director) shall be included as report appendices. Deviations from the protocol or Standard Operating Precedures not addressed by protocol amendment will be listed and their impact on the study noted

An audited draft report will be submitted to Sponsor for review and approval. Upon acceptance, five copies of the final report will be sent to Sponsor.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the relevant Good Laboratory Practice Regulations.

Should this be the last scheduled study with this test article, an archive sample will be taken at the time of the last dose preparation and sent immediately to Sponsor. The sample (approximately 2 gm for solids, 2 ml for liquids) will be placed in a labeled scintillation vial as directed in Protocol Appendix A. The vial will be tightly capped and sealed with tape to prevent accidental leakage of the material during transit, and will be accompanied by a sample transfer form.

Any unused test material will be disposed of by Microbiological Associates, Inc. unless otherwise requested by Sponsor.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EEC Legislation L251, vol. 27 (B.13, *Escherichia coli*-Reverse Mutation Assay; B.14, *Salmonella typhimurium*-Reverse Mutation Assay), September 1984; OECD Guideline 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay), May 1993; and EPA Health Effects Testing Guidelines, Subpart 798.5625 (The *Salmonella typhimurium* reverse mutation assay), Fed. Register, 40 CFR Part 700, July 1988 revision.

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? no If so, to which agency or agencies? n/a

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor require that samples of the Test Article dosing solutions be returned? no

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.

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.

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.

Yahagi, M., Nagao, Y., Seino, T., Sugimura, T. and Okada, M. (1977). Mutagenicities of N-nitrosamines on *Salmonella*. Mutation Research *48*:121-130.

14.0 APPROVAL

14.1 Generic Protocol Approval

Gary Roy for ALLIED-SIGNAL, INC.	Date
Richard H.C. San for MA	Date
MA Quality Assurance	Date

14.2. Study Specific Protocol Approval

Study Director	Date
MA Quality Assurance	Date

*To be completed upon authorization.

PROTOCOL APPENDIX A (one 2-page Appendix per Test Material)

TEST AND/OR CONTROL MATERIAL IDENTIFICATION AND HANDLING INFORMATION

This form is designed to specify test material identification, handling and disposition instructions and to provide procedures (if known) in case of accidental exposure to the material. As with all data, this information should be considered confidential and be available only to persons involved with the study or studies using this material.

PRINT OR TYPE THROUGHOUT
USE "NK" IF INFORMATION IS NOT KNOWN; USE "NA" IF NON-APPLICABLE

I. IDENTIFICATION

Test Material ID (Sample No.): FLEXAD (408-93C)
Batch or Lot No.: None CAS No.: 02897-60-1
Physical Description (form): Liquid Color: Clear
Purity (active ingredient): As received
Density: NK Flash Pt: 122°C (closed cup)
Vapor Pressure: NA pH: NK
Solubility: Soluble in acetone
Other: Will react with water
Specific gravity = 0.978

II. STORAGE INFORMATION

Material should be stored in:
Temperature-monitored room 65°-85°F: Yes
Refrigerator: No Freezer: No
Other storage conditions (specify): Cool dry place; protect from moisture

III. STABILITY

Length of time material is stable under conditions described above:
Assumed stable for duration of the study
Expiration Date: NK Indicated on Label: Yes; X No.

Preferred Vehicle: ~~Use N-23 for this study~~ ① Acetone

Stability in Vehicle: Unstable, should be tested soon after mixing with N-23

Analysis of neat material or material in vehicle (check if applicable):
____1. Will be required
_X_2. Will not be required
____3. Instructions for analysis will be provided
____4. Sample(s) is (are) to be submitted to sponsor for analysis

① per Sponsor request Nov 12/7/93

IV. KNOWN CHEMICAL HAZARDS

Reactivity/Flammability: Reacts with water, moisture
Temperature Range to Avoid: 122°C
Mixtures to Avoid: Water or moist air; oxidizing agents

PROTOCOL APPENDIX A (Cont'd.-Test Material ID (Sample No.): FLEXAD (408-93C)

V. KNOWN OR SUSPECTED TOXICITY HAZARDS

The toxicological properties of this material have not been thoroughly investigated.

Approximate rodent oral LD50: NK mL/Kg Species

Is material probable eye irritant: X Yes; No

Is material probable skin irritant: X Yes; No

Is material probably harmful if inhaled: X Yes; No

Other known toxicity: Mutagenic in Ames "Spot" Test

VI. HANDLING PRECAUTIONS

Use of protective clothing (laboratory coats), latex gloves, safety glasses and dust masks should be routine.

Routine precautions probably adequate: Yes; X No (see below)

Precautions in excess of routine (specify):

Emergency Treatment Procedures (if known) in case of accidental exposure are attached: Yes; X No

IN CASE OF EMERGENCY RELATED TO THIS SUBSTANCE, CONTACT:

Brendan J. Dunn (Person) of AlliedSignal Inc. (Company) at 201-455-6077 (Phone #)

VII. DISPOSITION

All materials will be returned to the sponsor (by arrangement) at the address below (IX):

Shipping Instruction (DOT Classification):

Nonregulated Research Sample

Materials not in original container (e.g., Archive samples) must be labeled with, at a minimum, sponsor's test material ID (Sample No.). Smaller containers (e.g., <500 mL) should have caps sealed with tape, and should be shipped inside an unbreakable secondary container (e.g., polyethylene bag) within the shipping carton.

VIII. SIGNATURE

Sponsor Signature: Brendan J. Dunn Date: 9-30-93

Brendan J. Dunn, M.S.

Phone Number: (202) 455-6077

IX. SPONSOR NAME AND ADDRESS:

Department of Toxicology
AlliedSignal, Inc.
101 Columbia Road
Morristown, N.J. 07962-1139

s:\tosdoc\byud\9-28-93a

APPENDIX III

Information for Japanese Regulatory Agencies

Report of Results of Reverse-Mutation Assay in Bacteria

1. General Items

Name of the new chemical subetance (IUPAC nomenclature)					
Other name	FLEXAD (408-93C)	Physico-chemical properties of the new chemical subetance	Molecular weight		
			Appearance at ordinary temperature		clear amber liquid
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)			Stability		
			Melting point		°C
			Boiling point		°C
			Vapor pressure		
			Partition coefficient		(°C)
Purity of the new chemical subetance tested	wt %		Solubility		
			Degree of solubility	Water	
				DMSO	
Name and concentration of impurities	wt %			Acetone	
				Other ( )	

[Remarks] Because physicochemical properties are reference materials, fill in spaces to extent possible.

1."Stability"-Fill in the stability for water, other solvents, heat, light, etc.
2."Vapor pressure"-Fill in the vapor pressure of the test substance at 25°C.
3."Partition coefficient"-Fill in the value, the temperature used and the name of the solvent used for the measurement.
4."Solubility"-Fill in such information as water-soluble, soluble in oil.
5."Degree of solubility"-Fill in the solubility at 25°C for each solvent.

2. 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 1992	The genetic markers for each culture are confirmed on the day of use
TA100			
TA1535			
TA1537			
WP2 *uvr*A (pKM101)	National Collection of Industrial and Marine Bacteria Aberdeen, Scotland	1 July 1987	
WP2 (pKM101)	National Collection of Industrial and Marine Bacteria Aberdeen, Scotland	19 February 1993	

(2) Storage (Encircle the applicable number, and fill in the relevant entries)

Freezing method	1. Subdivision (2.) Large quantity 3. Others ( )	
Storage temperature	≤-70°C	
Composition	Bacterial suspension	1.0 ml
	DMSO	0.09 ml
	Others	ml

3. S9 Mix

(1) Source of S9 (Encircle the applicable number, and fill in the relevant entries)

(1.) Made in-house	Prepared on	08/11/93, 10/21/93 and 11/10/93
2. Purchase	Supplier	
	Prepared on	(date)
	Purchased on	(date)
	Lot. No.	

(2) Storage Temperature, etc. of S9

Storage temperature	≤-70°C	Name and model of storage apparatus	So-Low, Model PR27-120

(3) Preparation of S9 (If purchased material, fill in spaces to extent possible)

Animal used		Inducing substance	
Species, Strain	Rattus norvegicus, Sprague Dawley	Name	Aroclor 1254
Sex	Male	Administration method	intraperitoneal
Age (in weeks)	6.5, 8.0 and 8.5 Weeks	Administration period and amount (g/kg-weight)	5 days 0.5 gm/kg body weight
Weight	168 to 192 g 157 to 201 g 173 to 197 g		

(4) Composition of S9 mix

Constituents	Amount in 1 ml S9 Mix	Constituents	Amount in 1 ml S9 Mix
S9	0.1 ml	NADPH	not applicable
$MgCl_2$	8 μmol	NADH	not applicable
KCl	33 μmol	Na-phosphate buffer (pH 7.4)	100 μmol
Glucose-6-phosphate	5 μmol	Others (NADP)	4 μmol
Glucose-6-phosphate dehydrogenase	not applicable		

4. *Positive Control Substance

(1) Positive control

Name	Manufacture	Lot No.	Grade	Purity (%)	Solvent used
9-Aminoacridine (9AAD)	Sigma Chemical Company	16F0679		98 %	DMSO
2-Aminoanthracene (2AA)	Sigma Chemical Company	58F3462	Practical		DMSO
Methyl methanesulfonate (MMS)	Aldrich Chemical Company	07118KT		99 %	DMSO
2-Nitrofluorene (2NF)	Aldrich Chemical Company	CK1511CJ		98 %	DMSO
Sodium azide (SA)	Sigma Chemical Company	110H0269	Practical		water
Sterigmatocystin (STM)	Sigma Chemical Company	39F4039	Not provided		DMSO

(2) Solvent

Name	Manufacture	Grade	Purity (%)
Dimethylsulfoxide (DMSO)	Fisher Scientific Company	Certified ACS	
Water	Gibco	deionized	

(3) Preparation and storage of positive control solution (Encircle the applicable number)

Prepare or Store	1. Prepare just before test (2.) Store subdivided solutions (Storage temp. -20 ± 5°C) 3. Others ( )

5. Preparation of Test Substance Solution (Encircle the applicable number, and fill in the revelant entries)

Solvent used	*Name	*Manufacture	*Lot No.	*Grade	*Purity (%)
	Acetone	Fisher Scientific Company	920807 and 933481	Certified ACS	
* Stability of test substance in the solvent	unknown				
* Reason to choose the solvent	Sponsor's request				
Method of suspension when test substance is difficult to dissolve	mixing immediately prior to dosing				
* Storage time and temp. from preparation to use for test	<1 hours 30 min. ambient °C				
* Conversion by purity	1. Yes (2.) No				

6. Conditions of Pre-culture (Encircle the applicable number, and fill in the relevant entries)

(1) Conditions

Nutrient broth	Name	*Manufacturer	*Lot No.
	Oxoid Nutrient Broth No. 2	Oxoid Ltd.	CH,-B,=26951477
Period of pre-culture	12 hours ± 1 hour		
* Storage time and temp. from inoculation to beginning of shaking culture	2 to 5 hours 0 min. ambient °C		
* Storage time and temp. from end of culture to use for test	<9 hours 30 min. 4±2 °C		
* Model and manufacturer of shaker	New Brunswick Scientific, model G-24		
* Method of shaking (shaking type, speed, etc.)	1. Reciprocal (2.) Rotary 3. Other 125 rev/min.		
* Culture vessel (shape, capacity)	shape: cylinder, 200 ml		
* Culture volume	50 ml		
* Volume of inoculum	1 colony		

(2) *Fresh cell

		Base-pair substitution type				Frameshift type		
		TA100	TA1535	WP2 *uvr*A (pKM101)	WP2 (pKM101)	TA98	TA1537	
Number of fresh cells ($X10^9$/ml)	Dose-finding study	1.8		6.5				
	Main study	0.5	0.7	2.1	1.6; 8.2	0.7	0.4	
	Confirmatory study	0.7	0.8	1.9	1.8; 7.3	0.8; 3.4	0.3	
Measurement Method		1. Conversion by OD value (2.) Dilution method 3. Other ( )						

7. Agar Plate Medium

(1) Top agar

Agar	Name	BBL Select
	Manufacturer	Becton Dickinson
	Lot No.	L3DYFP

(2) Minimum Glucose Agar (Encircle the applicable number, and fill in the relevant entries)

(1) Made in-house	Agar	Name	BBL Select
		Manufacturer	Becton Dickinson
		Lot No.	L3DYFP
	Volume of agar plate medium		25 ml
2. Purchased	Manufacturer		
	Prepared on	(date)	
	Purchased on	(date)	
	Lot No.		

8. Sterility test (Encircle the applicable number)

	Bacterial growth other than those used for test	
Test substance solution	1. Yes	(2.) No
S9 Mix	1. Yes	(2.) No

9. Test Method

(1) Test method (Encircle the applicable number)

(1.) Pre-incubation method
2. Plate method

(2) Test condition

		Pre-incubation method	Plate method
Composition	Bacterial suspension	0.1 ml	
	Test substance sol.	0.05 ml	
	Na-phosphate buffer	0.5 ml	
	S9 Mix (in case of metabolic activation method)	0.5 ml	
	Top agar solution	2.0 ml	
	Others ( )		
Pre-incubation	Temperature	37 ± 2 °C	
	Time	60 ± 2 min.	
Incubation	Temperature	37 ± 2 °C	
	Time	approximately 48 hours	

10. *Colony Counting (Encircle the applicable number, and fill in the relevant entries)

Counting Method	1. Manual 2. Colony counter (3.) Both
Reason to use both methods	Routinely all positive controls plates are counted with a colony counter. Routinely tester strain plates with revertant counts greater than 100 are counted with a colony counter and those less than 100 are counted manually. Plates with sufficient test article particulate to interfere with the colony counter are counted manually.
Automatic colony counter (model, manufacturer)	Artek Model 880, Artek Systems Corporation
Correction method for colony counter	1. Uncorrected (2.) Corrected (Actual manual count versus colony counter count)

11. Test Results

(1) Test results should be reported on the attached form.

(2) Judgement of the results

Judgement (Encircle one)	(positive)	negative
Reason for judgement and referential matters: Positive responses were observed with tester strains TA100, TA1535 and WP2 *uvr*A (pKM101) in the presence and absence of Aroclor-induced rat liver S9.		

(3) Referential matters

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

[Remark] "Referential matters" - Fill in the view etc. of the Study Director on the test results.

12. Others

Testing Facility	Name	Microbiological Associates, Inc.	
	Address	9900 Blackwell Road Rockville, Maryland 20850, U.S.A. Tel. (301) 738-1000	
Testing Facility Manager	Name	David Jacobson-Kram, Ph.D.	David Jacobson-Kram Signature
	Title	Vice President, Toxicology Group	
Filing and Storage	Name	Diane Gray	Diane Gray Signature
	Title	Secretary	
Manager of QAU	Name	Nona S. Karten, M.A.	Nona S. Karten Signature
	Title	Vice President, Regulatory Affairs/Quality Assurance	
Study Director	Name	Richard H.C. San, Ph.D.	Richard H.C. San Signature Experience: 24 years
	Title	Study Director, Bacterial Mutagenesis Studies	
Responsible Scientist	Name	Valentine O. Wagner, III, M.S.	Valentine O. Wagner, III Signature Experience: 13 years
	Title	Associate Study Director, Bacterial Mutagenesis Laboratory	
Test dates	_ from	10/13/93 to 01/10/94	
Study number	TD894.502070		

Table of Test Results

Name of Test Substance: FLEXAD (408-93C)

With (+) or without (-) S9 Mix	Test substance concentration (µg/plate)	Number of revertants (number of colonies/plate)						
		Base-pair substitution type				Frameshift type		
		TA100	TA1535	WP2 uvrA (pKM101)	WP2 (pKM101)	TA98	TA1537	
S9 Mix (-)	Solvent control	120 127 151 (133)	18 14 16 (16)	242 278 268 (259)	60 55 50 (55)	22 24 23 (23)	6 5 3 (5)	
	33	151 157 158 (155)	22 20 17 (20)	173 281 259 (238)	86 70 55 (70)	32 23 32 (29)	5 4 11 (7)	
	100	191 184 165 (180)	32 33 27 (31)	248 302 311 (287)	64 76 70 (71)	12 22 15 (16)	4 7 2 (4)	
	333	316 277 257 (283)	90 84 90 (85)	370 388 331 (363)	NC 59 63 (61)	22 25 25 (24)	6 3 9 (6)	
	1000	553 576 558 (562)	152 142 140 (145)	673 666 661 (667)	91 90 90 (90)	25 16 14 (18)	8 5 7 (7)	
	3333	0 0 651 (217)	90 34 175 (100)	698 991 1087 (922)	10 0 0 (3)	20 13 9 (14)	7 5 6 (6)	
	5000	0 0 0 (0)	0 0 127 (42)	0 0 0 (0)	5 7 0 (4)	0 0 0 (0)	0 0 0 (0)	
S9 Mix (+)	Solvent control	174 190 144 (166)	16 17 15 (16)	309 282 298 (296)	126 130 105 (120)	27 30 20 (26)	6 9 2 (6)	
	100	159 190 145 (165)	19 18 16 (18)	283 338 310 (314)	158 159 138 (152)	30 23 21 (25)	7 0 6 (4)	
	333	170 191 176 (179)	22 22 17 (20)	352 367 369 (359)	132 103 122 (119)	23 38 27 (30)	7 7 6 (7)	
	1000	260 257 267 (261)	56 68 58 (61)	443 412 423 (426)	129 134 MP (132)	49 27 34 (37)	6 5 8 (6)	
	2000	457 484 421 (454)	200 197 200 (199)	536 560 541 (546)	106 137 125 (123)	33 37 40 (37)	4 5 10 (6)	
	3333	427 571 602 (533)	67 196 208 (157)	830 816 719 (855)	136 151 124 (137)	37 33 31 (34)	6 4 8 (6)	
	5000	0 0 0 (0)	0 0 0 (0)	513 452 550 (505)	134 124 92 (117)	14 0 0 (5)	13 0 0 (4)	
Positive control not requiring S9 Mix	Name	SA	SA	MMS	MMS	2NF	9AAD	
	Concentration (µg/plate)	1.0	1.0	1000	1000	1.0	75	
	Number of colonies/plate	639 577 586 (601)	403 406 432 (414)	2214 1920 1862 (1928)	2249 2045 2087 (2127)	488 426 243 (386)	717 450 483 (550)	
Positive control requiring S9 Mix	Name	2AA	2AA	2AA	STM	2AA	2AA	
	Concentration (µg/plate)	1.0	1.0	10	100	1.0	1.0	
	Number of colonies/plate	1670 1920 2229 (1940)	107 108 101 (105)	2048 1796 1863 (1901)	257 268 MP (263)	1554 1674 1590 (1606)	252 330 278 (287)	

Notes:
1. When inhibition is found against growth of the bacteria, mark the applicable value with an asterisk.
2. Fill the average number of colonies in each concentration in the ( ).
3. "Number of revertants" - Fill in the observed value and average value in order beginning with the low concentrations of the test substance.

NC = plate did not receive an aliquot of tester strain MP = missing plate

Table of Test Results

Name of Test Substance: FLEXAD (408-93C)

With (+) or without (-) S9 Mix	Test substance concentration (µg/plate)	Number of revertants (number of colonies/plate)											
		Base-pair substitution type								Frameshift type			
		TA100		TA1535		WP2 *uvrA* (pKM101)		WP2 (pKM101)		TA98		TA1537	
S9 Mix (-)	Solvent control	NC 147 179	(163)	9 14 5	(9)	282 245 246	(251)	NC 85 92	(89)	18 19 25	(21)	9 6 6	(7)
	33	148 166 156	(157)	20 16 7	(14)	214 204 214	(211)	77 116 101	(99)	21 23 21	(22)	6 9 4	(6)
	100	179 200 NC	(190)	20 24 26	(23)	275 245 219	(246)	62 73 79	(71)	27 18 19	(21)	6 8 5	(6)
	333	287 306 285	(293)	56 73 45	(58)	336 362 322	(340)	57 86 91	(78)	22 32 31	(28)	9 7 NC	(8)
	1000	412 321 479	(404)	123 120 105	(116)	497 512 540	(516)	107 100 88	(98)	21 27 34	(27)	6 7 6	(6)
	3333	0 0 0	(0)	0 0 127	(42)	741 712 498	(650)	0 0 0	(0)	22 0 0	(7)	0 0 0	(0)
	5000	0 0 0	(0)	0 0 0	(0)	0 0 0	(0)	0 0 0	(0)	0 0 0	(0)	0 0 0	(0)
S9 Mix (+)	Solvent control	160 142 174	(159)	15 26 19	(20)	214 303 283	(267)	131 130 85	(115)	28 31 33	(31)	7 9 4	(7)
	100	180 167 165	(171)	14 15 26	(18)	240 280 304	(275)	104 144 129	(126)	41 24 26	(30)	4 7 3	(5)
	333	188 226 158	(190)	23 28 22	(24)	313 282 278	(295)	141 134 134	(136)	31 37 37	(35)	17 4 4	(8)
	1000	240 249 270	(253)	60 57 63	(60)	224 361 359	(315)	131 133 145	(136)	35 41 29	(35)	10 10 2	(7)
	2000	451 340 353	(381)	176 180 178	(178)	608 465 572	(548)	89 122 83	(98)	45 43 43	(44)	7 3 8	(6)
	3333	714 612 641	(656)	337 328 131	(265)	814 399 842	(685)	6 0 27	(11)	13 55 44	(37)	2 6 1	(3)
	5000	0 0 892	(297)	500 388 340	(410)	1075 876 787	(913)	0 0 0	(0)	0 0 0	(0)	5 7 5	(6)
Positive control not requiring S9 Mix	Name	SA		SA		MMS		MMS		2NF		9AAD	
	Concentration (µg/plate)	1.0		1.0		1000		1000		1.0		75	
	Number of colonies/plate	531 588 634	(584)	600 561 602	(588)	2465 2423 2472	(2453)	1385 1826 1757	(1649)	998 1481 1547	(1135)	790 729 724	(748)
Positive control requiring S9 Mix	Name	2AA		2AA		2AA		STM		2AA		2AA	
	Concentration (µg/plate)	1.0		1.0		10		100		1.0		1.0	
	Number of colonies/plate	1350 1348 1447	(1382)	138 144 164	(149)	2038 1898 1995	(1977)	253 245 240	(246)	1185 1188 1233	(1199)	126 412 827	(455)

Notes:

1. When inhibition is found against growth of the bacteria, mark the applicable value with an asterisk.
2. Fill the average number of colonies in each concentration in the ( ).
3. "Number of revertants" - Fill in the observed value and average value in order beginning with the low concentrations of the test substance.

NC = Plate did not receive an aliquot of tester strain

Table of Test Results (Potency)

Name of Test Substance: FLEXAD (408-93C)

	Test Strain	-S9 mix		+S9 mix	
		Potency (revertants/mg)	Dose (µg/plate)	Potency (revertants/mg)	Dose (µg/plate)
Dose-finding study	TA100	368	1000	208	5000
	TA1535				
	WP2 *uvr*A (pKM101)	431	1000	210	3333
	TA98				
	TA1537				
	WP2 (pKM101)				
Main study	TA100	429	1000	110	3333
	TA1535	129	1000	92	2000
	WP2 *uvr*A (pKM101)	199	3333	108	3333
	TA98	182	33	6	2000
	TA1537	2	1000	3	333
	WP2 (pKM101)	35	1000	320	100
Confirmatory study	TA100	241	1000	149	3333
	TA1535	107	1000	78	5000
	WP2 *uvr*A (pKM101)	120	3333	129	5000
	TA98	21	333	7	2000
	TA1537	3	333	3	333
	WP2 (pKM101)	303	33	21	1000

Potency (revertants/mg) = maximum net revertants/concentration (mg); maximum net revertants = maximum revertant count - solvent control revertant count.

If the maximum revertant count is observed at more than one dose level, the higher concentration (or highest concentration, if applicable) is used in the potency computation.

If the maximum revertant count is equal to or less than the vehicle control value, the potency is indicated as zero at the highest dose level tested.