

**REPORT DOCUMENTATION PAGE**

*Form Approved  
OMB No. 0704-0188*

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

**PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 20-03-1996		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1-30-1996 to 3-20-1996	
4. TITLE AND SUBTITLE Salmonella Typhimurium Microsome Reverse Mutation Assay				5a. CONTRACT NUMBER DAAD 05-91-C-0018	
				5b. GRANT NUMBER NA	
				5c. PROGRAM ELEMENT NUMBER ---	
				5d. PROJECT NUMBER ILS-A073-001	
6. AUTHOR(S) Paul W. Andrews Raymond R. Tice Diane Satterfield				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Integrated Laboratory System 800-12, Capital drive Drum, NC 27713				8. PERFORMING ORGANIZATION REPORT NUMBER --	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S Army Center for Health Promotion & Preventive Medicine Aberdeen Proving Ground, MD 21010				10. SPONSOR/MONITOR'S ACRONYM(S) USAPHC/AIPH/TOX	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) --	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES --					
14. ABSTRACT The results of the Ames Salmonella typhimurium assay on trifluoromethane showed a negative mutagenic response in all five strains in the presence and absence of metabolic activation.					
15. SUBJECT TERMS Ames Assay, Salmonella Mutegenicity Assay, FE-13, Trifluoromethane,					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 30	19a. NAME OF RESPONSIBLE PERSON Dr. Gunda Reddy
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (Include area code) 410-436-3980

# Integrated Laboratory Systems

## STUDY TITLE

Salmonella Typhimurium Microsome Reverse Mutation Assay

## Project No.

ILS A073-001

## Contract No.

DAAD05-91-C-0018

## Test Substance

FE-13

## ILS Repository No.

96-01

## Final Report Date

March 20, 1996

## Sponsor

U.S. Army CHPPM  
Bldg. E-2100  
Aberdeen Proving Ground, MD 21005

## Testing Facility

Integrated Laboratory Systems  
800-12 Capitola Drive  
Durham, NC 27713

---

P.O. Box 13501  
Research Triangle Park, NC 27709

## QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: A073-001  
Test Substance: FE-13  
ILS No.: 96-01  
Study Title: Salmonella Typhimurium Microsome Reverse Mutation Assay

This study was inspected by one or more persons of the Quality Assurance Unit of Integrated Laboratory Systems, Research Triangle Park, NC, and written status reports were submitted on the following dates:

<u>Inspection/Audit</u>	<u>Date Performed</u>	<u>Date Reported to Study Director/Management</u>
Study Protocol:	1-30-96	1-30-96; 1-31-96
Preparation of S9 mix and Plate Incorporation	2-20-96	2-20-96; 2-21-96
Plate Counting	2-23-96	2-26-96; 2-26-96
Data audit	3-14-96	3-15-96; 3-20-96
Report audit:	3-14-96	3-15-96; 3-20-96

Kaye Cummings  
Kaye Cummings, B.S.  
Quality Assurance Auditor

3/20/96  
Date

CERTIFICATION OF GOOD LABORATORY PRACTICE

ILS Project No.: A073-001  
Study Title: Salmonella Typhimurium Microsome Reverse Mutation Assay  
Test Substance: FE-13  
ILS No.: 96-01

This study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 792) except that the purities of the positive control substances were not provided by the suppliers.

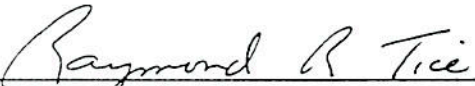
  
\_\_\_\_\_  
Paul W. Andrews, M.S.  
Study Director

  
\_\_\_\_\_  
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: A073-001  
Study Director: Paul W. Andrews, M.S.  
Study Title: Salmonella Typhimurium Microsome Reverse Mutation Assay  
Test Substance: FE-13

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAD05-91-C-0018 is complete, accurate, and complies with all requirements of the contract.

  
\_\_\_\_\_  
Raymond R. Tice, Ph.D.  
Vice President, Research & Development

3/20/96  
Date

## TABLE OF CONTENTS

	<u>Page</u>
1.0 Study Title .....	1
2.0 Study Identification .....	1
3.0 Purpose of the Study .....	1
4.0 Names and Addresses of Sponsor and Testing Facility .....	1
4.1 Sponsor .....	1
4.2 Testing Facility .....	1
5.0 Study Dates .....	1
6.0 Primary Study Personnel .....	2
7.0 Test substance .....	2
7.1 Identification .....	2
7.2 Physical description and composition .....	2
7.3 Properties of the Test substance .....	2
8.0 Test System .....	2
8.1 Test System Justification .....	2
8.2 Source and Types of Salmonella Strains .....	2
9.0 Experimental Design .....	3
9.1 S9 Activation System .....	3
9.2 Dose Levels .....	3
9.2 Selection of Positive Controls .....	3
9.4 Statistical Analysis .....	3
10.0 Criteria for Determination of a Valid Test .....	4
11.0 Criteria for a Positive Response .....	4
12.0 Records to be Maintained .....	4
13.0 Quality Assurance .....	5
14.0 Test Substance Disposition .....	5
15.0 Results .....	5
15.1 Chemicals and Reagents .....	5
15.2 Toxicity Test .....	5
15.3 Mutation Assay .....	5
16.0 Conclusion .....	6
17.0 References .....	6
Tables 1 & 2 .....	8
Appendix 1: Protocol & Amendments .....	10

## FINAL REPORT

---

### 1.0 Study Title:

*Salmonella Typhimurium* Microsome Reverse Mutation Assay

### 2.0 Study Identification:

ILS Project No. A073-001  
Contract No. DAAD05-91-C-0018

### 3.0 Purpose of the Study:

The objective of this study is to investigate the mutagenic potential of the test substance in five strains of *Salmonella typhimurium* in the presence and absence of metabolic activation.

### 4.0 Names and Addresses of Sponsor and Testing Facility:

4.1 Sponsor: U.S. Army CHPPM  
Bldg E-2100  
Aberdeen Proving Ground, MD 21005

4.2 Testing Facility: Integrated Laboratory Systems

Shipping Address: 800-12 Capitola Drive  
Durham, NC 27713

Mailing Address: P.O. Box 13501  
Research Triangle Park, NC 27709

### 5.0 Study Dates:

Study Initiation Date: January 31, 1996  
Experimental Start Date: February 7, 1996  
Experimental Termination Date: March 4, 1996  
Study Completion Date: March 20, 1996

6.0 Primary Study Personnel:

Paul W. Andrews, M.S., Study Director  
Raymond Tice, Ph.D., Vice President, Research & Development  
Diane Satterfield A.S., Research Assistant

7.0 Test substance:

7.1 Identification: FE-13

7.2 Physical description and composition: gas

7.3 Properties of the Test substance:

7.3.1 Compound Characterization: Determination of test substance stability and test substance characteristics is the responsibility of the sponsor. Information on the test substance including its method of synthesis, analysis, physicochemical characteristics, and bulk stability is retained on file by the sponsor.

7.3.2 Storage Conditions: The test substance was stored at room temperature in the ILS Chemical Repository. Stability under these conditions has been demonstrated by the Sponsor and documentation is on file with them. Normal safety precautions appropriate for potential mutagens were followed when handling the test substance. A material safety data sheet was not provided.

8.0 Test System:

8.1 Test System Justification: The Salmonella/Ames test is a simple and sensitive bacterial test for detecting chemical mutagens (1-3). In this test, the test substance was assayed with several specially constructed mutants for *Salmonella typhimurium* selected for sensitivity and specificity in being reverted from histidine requirements back to prototrophy by a wide variety of mutagens (4-6). To increase the scope of sensitivity of the test, a rat liver homogenate (S9) was directly applied to the plates and thus mutagens requiring metabolic activation can also be detected.

8.2 Source and Types of Salmonella Strains: The sponsor specified the five strains to be used for conducting the assay. *Salmonella typhimurium* tester strains (ie., TA98, TA100, TA1535, TA1537, and TA1538) were obtained through the courtesy of Dr. B. N. Ames, Biochemistry Department, University of California at Berkeley. Single colony isolates obtained from

these original cultures were regrown in nutrient broth and maintained as permanent cultures at approximately -70°C in 9% dimethylsulfoxide (DMSO). The permanent cultures were simultaneously checked for both phenotypic and genotypic characteristics

## 9.0 Experimental Protocol

The pour-plate incorporation technique, modified for a gas phase test substance, was used with freshly grown bacterial cultures. Simply, 2 ml of molten top agar was mixed with with 100  $\mu$ l aliquot of the bacterial culture. Trace amounts of histidine (0.05 mM) and biotin (0.05 mM) were added to the top agar to allow a few divisions of the auxotrophic bacterial strains. To incorporate metabolic activation, 0.5 ml of S9 mix (S9 fraction with an NADP generating co-factor mixture) was added where appropriate or 0.5 ml PBS for the nonactivated portion. The contents of the test tube were properly swirled on a vortex mixer and poured over previously prepared Vogel-Bonner minimal medium plates. Plates were exposed to the test agent in triplicate in sealed Tedlar bags and incubated at  $37\pm 1^\circ\text{C}$  for 24 hours. At the end of the exposure period, plates were removed from the bags and incubated for an additional 24-48 hours before counting revertant colonies.

- 8.1 S9 Activation System: Metabolic activation was incorporated into the assays by the addition of a post mitochondrial supernatant (S9 fraction) prepared from rat liver homogenates of male Sprague Dawley rats induced with Aroclor 1254.
- 8.2 Dose Levels: A toxicity test was conducted on FE-13 with and without S9 activation at 10, 50, and 100% per plate using strain TA100. As directed by the sponsor, air was the solvent of choice. Toxicity was determined by examining the plates for the thinning of bacterial lawn, microcolony formation, or complete growth inhibition.
- 8.3 Selection of Positive Controls: Positive controls were selected by their specificity to revert tester strains. The missense tester strains (TA1535 and TA100) were tested with sodium azide (2.5 and 5.0  $\mu\text{g}/\text{plate}$ ) while the frameshift strain (TA1538 and TA98) were tested with 2-nitrofluorene (3 and 6  $\mu\text{g}/\text{plate}$ ). The intercalating agent, 9-aminoacridine (25, 37.5, and 50  $\mu\text{g}/\text{plate}$ ), was used to test strain TA1537. 2-aminoanthracene (2.5, 3.0, 5.0, 6.0, 6.25, or 12.5  $\mu\text{g}/\text{plate}$ , depending on the strain) was used with all tester strains to evaluate S9 activation. DMSO was the solvent used for all positive control dilutions.
- 8.4 Data Analysis: Revertant colonies were counted on an Artek autocount

colony counter or hand-counted. Before counting, the plates were verified for confluent growth of the bacterial lawn. Plates lacking background bacterial lawn or with microcolonies were recorded as such on data sheets but were not used in data analysis. Data were analyzed using the "Salmonella Assay Data Management and Analysis Software," Version 1.0; however, the study director made the final interpretation.

#### 10.0 Criteria for Determination of a Valid Test

The following criteria should be satisfied for accepting test data:

1) The mean solvent control data should be within acceptable ranges, as follows:

TA1535, TA1537, and TA1538: 4-40 revertants/plate  
TA98: 20-65 revertants/plate  
TA100: 50-250 revertants/plate

2) The positive controls should exhibit a 3-fold (TA1535, TA1537, TA1538) or 2-fold (TA98, TA100) increase over solvent control data.

#### 11.0 Criteria for a Positive Response

A test substance was regarded as mutagenic if the mean induced revertant number equaled 3.0 or more the mean solvent control number of colonies for strains TA1535, TA1537, and TA1538, and 2.0 or more for strains TA98 and TA100. This increase must be accompanied by a dose-dependent response to increasing test substance concentrations. A sample was considered weakly positive if there was no dose response but one or more doses exhibited a doubling/tripling over solvent controls or if there was a dose response but no doses exhibited an appropriately high number of revertants.

#### 12.0 Records to be Maintained:

Raw data sheets, statistical printouts, data diskettes, and a copy of the final report will be archived at ILS, 800-12 Capitola Dr., Durham, NC 27713 at the completion of the study until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. After this time all items will be transferred to the sponsor for archiving.

13.0 Quality Assurance:

Quality assurance inspections of ongoing studies was conducted at intervals adequate to assure the integrity of the study. At least one critical phase was inspected for each study. An audit of the final report was conducted to verify that reported values are supported by the data and that no conditions are present that might affect the outcome of the study.

14.0 Test Substance Disposition:

Any unused test substance and a log accounting for all test substance use will be returned to the sponsor upon completion of the contract.

15.0 Results:

15.1 Chemicals and Reagents

The following chemicals and reagents were obtained from commercial sources (purity not provided) as follows:

<u>Chemical</u>	<u>Lot Number</u>	<u>Source</u>
Compressed air	N/A	Air Products
Biotin	85H0316	Sigma
Histidine	26F-0649	Sigma
Dimethylsulfoxide	952812	Fisher
Magnesium chloride	44H0170	Sigma
Potassium chloride	15H0530	Sigma
Glucose-6-Phosphate	12H3805/85H3851	Sigma
Sodium Phosphate	53H02645	Sigma
Sodium Azide	121H3804	Sigma
2-Nitrofluorine	JF01215AZ	Aldrich
9-Aminoacridine	56F0316	Sigma
2-Aminoanthracene	121H3804	Sigma
Rat liver S9	0588, 0599	Mol. Tox.

15.2 Toxicity Test: In the toxicity test, FE-13 (ILS # 96-01) exhibited an approximate 50% reduction in the number of revertants per plate in both the presence and absence of metabolic activation at the highest dose tested (100%). Hence, the top dose for the assay was set at 100% FE-13/plate in both the presence and absence of S9 activation.

15.3 Mutation Assay: The test substance was evaluated for mutagenic activity

in the Salmonella typhimurium plate incorporation assay with five tester strains. The sample was tested directly and in the presence of liver homogenates (S9 fraction) from rats treated with Aroclor 1254. Concurrent positive and solvent controls were run along with five dose points of the test sample. Air was used as the solvent in preparing all gas concentrations. Dimethylsulfoxide was used as the solvent in preparing positive control stock solutions. A 100% nitrogen control was included in order to assess the effects of oxygen deprivation. All tests were run in triplicate plates. The doses tested were 100.0, 75.0, 50.0, 25.0, and 10.0% FE-13 per plate in both the presence and absence of S9 activation.

The experimental results obtained with FE-13 are presented in Table 1 according to strain as the number of mean his+ revertants per plate. Individual plate counts for each test dose and control are presented in Table 2. The results of the assay on the test substance FE-13 indicate no significant mutagenic response in all five strains both in the presence and absence of metabolic activation. Strain TA98 at 25% FE-13 exhibited a mean that was 2.1 times the mean of the 100% air control. However, this was due to a single high plate count and is not considered biologically relevant. The positive controls exhibited a significant increase in mutant colonies in all strain both with and without S9. A reduction in the number of revertants per plate was observed with both 100% FE-13 and 100% nitrogen in strains TA1538, TA98, and TA100 both with and without S9 indicating this effect was most likely due to oxygen deprivation and not test substance associated toxicity.

## 16.0 Conclusion

The results of the assay on FE-13 (ILS # 96-01) indicate a negative mutagenic response in all five strains both in the presence and absence of metabolic activation.

## 17.0 References

1. Haworth, S., T. Lawlor, K. Mortelmans, W. Speck, and E. Zeiger (1983). Salmonella mutagenicity test results for 250 chemicals. Environ. Mutagen. Suppl. 1: 3-142.
2. Ames, B.N. (1971). Detection of chemical mutagens with enteric bacteria. In: Chemical Mutagens, A. Hollaender (ed.), Vol. 1, pp. 267-282. New York, Plenum Press.
3. Ames, B.N., F.D. Lee, and W.E. Durston (1973). An improved bacteria test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. USA 70(3): 782-786.

4. 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(12): 5135-5139.
5. Ames, B.N., J. McCann, and E. Yamasaki (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutat. Res.* 31: 347-374.
6. Maron, D.M. and B.N. Ames (1983). Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* 113: 173-215.

TABLE 1. Salmonella typhimurium Mutagenesis Assay of FE-13 (ILS No. 96-01)

		MEAN HIS+ REVERTANTS/PLATE									
		TA1535		TA1537		TA1538		TA98		TA100	
DOSE (%)	S9	mean	std	mean	std	mean	std	mean	std	mean	std
0.0	-	18.0 +/- 1.73	13.0 +/- 3.61	26.3 +/- 3.79	24.3 +/- 5.13	209.0 +/- 20.30					
10.0	-	19.7 +/- 2.52	14.0 +/- 2.65	26.0 +/- 9.85	41.0 +/- 14.00	173.0 +/- 6.93					
25.0	-	22.0 +/- 3.61	12.3 +/- 5.86	22.7 +/- 3.79	51.7 +/- 22.81	174.7 +/- 13.05					
50.0	-	26.7 +/- 4.04	10.7 +/- 3.21	27.0 +/- 1.73	43.0 +/- 11.79	163.0 +/- 22.27					
75.0	-	15.0 +/- 1.00	9.0 +/- 3.46	17.0 +/- 3.61	22.7 +/- 12.50	149.3 +/- 20.23					
100.0	-	19.7 +/- 4.51	8.0 +/- 2.00	5.0 +/- 5.66	15.0 +/- 1.73	150.0 +/- 14.00					
100 N2	-	21.0 +/- 4.58	9.3 +/- 3.06	13.0 +/- 6.24	17.3 +/- 5.51	96.0 +/- 28.16					
DMSO	-	22.3 +/- 1.53	12.3 +/- 0.58	23.7 +/- 1.53	41.0 +/- 2.65	208.3 +/- 15.89					
POS	-	820.0 +/- 63.00	392.7 +/- 206.08	229.7 +/- 47.96	762.3 +/- 58.07	1040.3 +/- 108.74					
0.0	+	11.0 +/- 8.00	18.0 +/- 4.24	10.3 +/- 6.11	36.0 +/- 13.53	187.0 +/- 12.49					
10.0	+	6.7 +/- 2.08	11.0 +/- 1.00	10.3 +/- 11.15	30.3 +/- 6.66	157.3 +/- 12.58					
25.0	+	8.3 +/- 3.06	10.3 +/- 0.58	19.3 +/- 10.79	37.7 +/- 4.93	136.7 +/- 12.06					
50.0	+	13.0 +/- 2.65	10.3 +/- 0.58	23.7 +/- 4.93	35.0 +/- 10.82	152.7 +/- 12.22					
75.0	+	10.0 +/- 2.65	17.3 +/- 7.23	19.0 +/- 4.00	27.3 +/- 2.08	132.7 +/- 7.37					
100.0	+	10.7 +/- 1.15	20.0 +/- 1.00	4.0 +/- 1.00	18.7 +/- 8.50	111.3 +/- 4.04					
100 N2	+	13.0 +/- 2.65	18.0 +/- 5.20	2.0 +/- 0.00	19.3 +/- 4.73	124.0 +/- 14.00					
DMSO	+	9.7 +/- 0.58	17.3 +/- 4.51	13.0 +/- 14.80	44.3 +/- 8.33	202.3 +/- 5.86					
POS	+	239.3 +/- 25.70	358.3 +/- 72.82	630.7 +/- 98.36	1555.3 +/- 53.90	2040.7 +/- 111.85					

Data presented as the mean of triplicate plates +/- the standard deviation

TABLE 2. Salmonella typhimurium Mutagenesis Assay of FE-13 (ILS No. 96-01)

DOSE (%)	S9	his+ revertants/plate														
		TA1535			TA1537			TA1538			TA98			TA100		
0.0	-	17	17	20	14	16	9	29	28	22	20	30	23	191	231	205
10.0	-	22	20	17	17	12	13	23	18	37	25	51	47	169	181	169
25.0	-	21	26	19	10	19	8	20	21	27	78	39	38	179	160	185
50.0	-	23	31	26	7	13	12	28	25	28	30	46	53	187	159	143
75.0	-	16	14	15	7	7	13	14	21	16	37	17	14	126	162	160
100.0	-	24	15	20	6	10	8	9	NG	1	13	16	16	156	160	134
100 N2	-	22	25	16	6	12	10	6	15	18	17	23	12	128	75	85
DMSO	-	22	24	21	13	12	12	24	22	25	38	42	43	190	217	218
POS	-	775	793	892	223	622	333	281	222	186	759	822	706	1165	965	991
0.0	+	19	3	11	21	NG	15	5	9	17	35	23	50	197	173	191
10.0	+	6	9	5	11	10	12	23	6	2	23	32	36	169	144	159
25.0	+	5	11	9	10	11	10	7	27	24	32	41	40	148	124	138
50.0	+	12	11	16	10	11	10	18	26	27	26	47	32	142	150	166
75.0	+	7	11	12	9	22	21	19	15	23	25	28	29	130	141	127
100.0	+	12	10	10	20	19	21	3	5	4	22	25	9	109	116	109
100 N2	+	15	10	14	21	21	12	NG	2	NG	14	21	23	108	130	134
DMSO	+	10	10	9	13	17	22	30	6	3	47	35	51	200	209	198
POS	+	243	263	212	420	278	377	589	743	560	1544	1508	1614	1918	2137	2067

NG = no growth

N2 = nitrogen gas

POS = positive control

DMSO = dimethylsulfoxide solvent control for POS

APPENDIX 1

PROTOCOL & AMENDMENTS

# INTEGRATED LABORATORY SYSTEMS

---

## STUDY PROTOCOL

### 1.0 Study Title:

Salmonella Typhimurium Microsome Reverse Mutation Assay

### 2.0 Study Identification:

ILS Project No. A073-001  
Contract No. DAAD05-91-C-0018

### 3.0 Purpose:

The objective of this study is to investigate the mutagenic potential of the test substance in five strains of Salmonella typhimurium in the presence and absence of metabolic activation.

### 4.0 Management of the Study:

4.1 Sponsor - U. S. Army CHPPM  
Bldg E-2100  
Aberdeen Proving Ground, MD 21005

#### 4.2 Testing Laboratory:

Mailing Address - Integrated Laboratory Systems  
P. O. Box 13501  
Research Triangle Park, NC 27709

Shipping Address - Integrated Laboratory Systems  
800-12 Capitola Drive  
Durham, NC 27713

4.3 Project Officer - LeRoy Metker

4.4 Study Director - Paul W. Andrews, M.S.

### 5.0 Project Schedule:

Proposed Experimental Start Date: February 5, 1996

Proposed Experimental Termination Date: April 5, 1996

## ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

---

### 6.0 Test Substance:

6.1 Identification: FE-13 (ILS # 96-01)

6.2 Physical description: gas

6.3 Properties of the Test Substance:

Compound Characterization: Determination of test substance stability and test substance characteristics is the responsibility of the sponsor. Information on the test substance including its method of synthesis, analysis, physicochemical characteristics, and bulk stability is retained on file by the sponsor.

Storage Conditions: The test substance will be stored at room temperature in the ILS Chemical Repository. Stability under these conditions has been demonstrated by the sponsor and documentation is on file with them. Normal safety precautions appropriate for potential mutagens will be necessary when handling the test substance.

### 7.0 Test System:

7.1 Rationale: The Salmonella/Ames test is a simple and sensitive bacterial test for detecting chemical mutagens (1-3). In this test, the test substance will be assayed with several specially constructed mutants for *Salmonella typhimurium* selected for sensitivity and specificity in being reverted from histidine requirements back to prototrophy by a wide variety of mutagens (4-6). To increase the scope of sensitivity of the test, a rat liver homogenate (S9) is directly applied to the plates and thus mutagens requiring metabolic activation can also be detected.

7.2 Source and Types of Salmonella Strains: The sponsor has specified the five strains to be used for conducting the assay. *Salmonella typhimurium* tester strains (ie. TA98, TA100, TA1535, TA1537, and TA1538) were obtained through the courtesy of Dr. B. N. Ames, Biochemistry Department, University of California at Berkeley. Single colony isolates obtained from these original cultures were regrown in nutrient broth and maintained as permanent cultures at approximately -70°C in 9% dimethylsulfoxide (DMSO). The permanent cultures were simultaneously checked for both phenotypic and genotypic characteristics as outlined below.

ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

Table 1. Phenotypic Verification of Salmonella Strains

Strain	Selective Medium				
	Minimal	Crystal Violet	Ampicillin	Tetracycline	UV
TA98	S	S	R	-	S
TA100	S	S	R	-	S
TA1535	S	S	S	-	S
TA1537	S	S	S	-	S
TA1538	S	S	S	-	S

S = sensitive (no growth); R = resistant (growth)

7.3 Growth of Tester Strains: One well isolated colony from the master plate is removed, inoculated in 20 ml nutrient broth, and grown overnight in a shaker incubator (16-20 hours at  $37\pm 1^\circ\text{C}$ ). Alternately, a frozen aliquot of the tester strain is thawed and immediately added to nutrient broth for overnight growth in a shaker incubator (16-20 hours at  $37\pm 1^\circ\text{C}$ ). On the day of the test, the density of the cultures is visually checked. Any culture exhibiting poor growth is not used.

7.4 Strain Marker Verification: For each master plate or frozen aliquot, the strains used are tested for the presence of the *rfa* and *uvrB* mutations. The *rfa* mutation permits large molecules such as crystal violet to enter the bacteria. Crystal violet inhibits bacterial growth within its diffusion zone. At the same time, strains are tested for the ampicillin-resistant R-factor. To test these characteristics, all strains are streaked in straight lines on two nutrient agar plates. On one extreme of one streak, a solution of crystal violet is placed. On the other extreme, antibiotic discs are sterilely placed. There should not be any growth in the crystal violet zone for all strains. In the ampicillin resistance test, only TA100 and TA98 should be observed growing in the vicinity of the ampicillin discs. On the second plate, half of the streaks are exposed to ultraviolet light for 5 to 10 seconds. There should not be any growth on the UV exposed side for all strains. Failure of a strain to exhibit any one of the expected phenotypic manifestations will render experimental results unacceptable.

7.5 Preparation of Bacteriological Media and Stock Solutions: The bacteriological media and pertinent stock solutions for performing Salmonella mutagenesis tests are listed below.

## ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

1. 50X VB Salts
2. Bottom Agar - VB Plates
3. Nutrient Agar Plates
4. Top Agar
5. Nutrient Broth
6. Biotin/Histidine (0.5 mM)
7. Cofactor Stocks:
  - a. KCl (1.65M)
  - MgCl<sub>2</sub> (0.4M)
  - b. Glucose-6-phosphate (1.0M)
  - c. NADP (0.1M)
  - d. Na<sub>2</sub>HPO<sub>4</sub> (0.2M)

### 8.0 Experimental Design

Samples are tested in triplicate for mutagenicity at five dose points. The high dose is generally limited by cellular toxicity, solubility or availability of the sample but will not exceed 10  $\mu$ l/plate (liquid), 10 mg/plate (solids), or 100%/plate (gas). The four remaining doses will be approximately 1/2, 1/10, 1/20, and 1/100th the high dose selected. Metabolic activation is included by adding Aroclor 1254-induced rat liver S9 fractions. Concurrent positive and solvent controls are run in all mutagenicity experiments (see Table 2).

Table 2. Positive Control Mutagens for the Salmonella Assay

<u>Strain</u>	<u>S9</u>	<u>Positive Control</u>	<u>CAS No.</u>	<u>Dose (ug/plate)</u>
TA98	-	2-Nitrofluorene	607-57-8	3.0, 6.0
TA100	-	Sodium Azide	26628-22-8	2.5, 5.0
TA1535	-	Sodium Azide	26628-22-8	2.5, 5.0
TA1537	-	9-Aminoacridine	90-45-9	25, 50
TA1538	-	2-Nitrofluorene	607-57-8	3.0, 6.0
all strains	+	2-Aminoanthracene	613-13-8	2.5 - 12.5

8.1 Preparation of Test Substance and Positive Controls: Information on the chemical and physical characteristics, stability, purity, solubility, storage condition and safety precautions (if available) are supplied by the sponsor. As directed by the sponsor, ambient air is the solvent of choice. Test substance doses are prepared fresh for each test unless otherwise specified. Solutions of positive control substances are prepared in disposable, sterile glass tubes within 60 minutes of administering each

## ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

dose and stored at ambient conditions until used. The tubes are placed in racks clearly labeled with the strain, ILS #, dose level, and a "+" or "-" S9 designation. Solutions of 9-aminoacridine, sodium azide, 2-nitrofluorene, and 2-aminoanthracene are prepared in DMSO and can be stored frozen for up to one year.

8.2 Preparation of S9 Mix: Immediately prior to mutagenesis testing, frozen aliquots of S9 are thawed and maintained at 1-6°C for no more than one hour. The S9 is mixed with cofactors to provide an NADP-generating system. This mixture, referred to as the "S9 mix", is prepared at a final concentration of 10% S9 fraction to the final volume of S9 mix. Cofactor concentrations in the S9 mix are 8 mM MgCl<sub>2</sub>, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP and 100 mM HPO<sub>4</sub>, pH 7.4.

8.3 Dose Range Selection: A toxicity test will be conducted with TA100, with and without S9 activation, to select the dose range. An aliquot of freshly grown bacterial culture (0.1 ml) will be mixed with 2.0 ml of top agar containing histidine and biotin. Plates are exposed to the test agent in triplicate (3 doses at 100, 50, and 10% FE-13, 100% nitrogen, and 100% air) in Tedlar bags and incubated at 37±1°C for 24 hours. At the end of the exposure period, plates will be removed from the bags and incubated for an additional 24 hours before counting revertant colonies. Exact doses for the assay, when determined, will be addressed in a protocol amendment.

### 8.4 Mutagenesis Assay:

1. Prepare the sterile S9 mix and store at 1-6°C for up to one hour prior to dosing.
2. Melt the top agar and supplement with 10 ml of 0.5 mM biotin/histidine solution per 100 ml agar.
3. Place labelled, sterile, capped tubes at 45-47°C and, following the order presented, supplement each tube with:
  - 2.0 ml supplemented top agar
  - 0.1 ml overnight bacterial culture
  - 0.5 ml S9 mix (for activated test) or 0.5 ml PBS (for nonactivated test)
4. Vortex the mixture gently and pour onto a minimal medium bottom agar plate, tilt to allow even distribution over the surface of the agar. Allow plates to harden in the hood at room temperature.
5. Insert inverted plates into Tedlar bags (one bag per dose) and seal by taping the open end shut, folding the sealed end 2-3 times, and clamping tight

## ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

with clips. Remove residual air from the bags with a vacuum pump. Inflate bags with the desired test substance dose and incubate entire bag at  $37 \pm 1^\circ\text{C}$ .

6. After 24 hours incubation, remove Tedlar bags from the incubator and open the bags in a hood to vent gas and remove plates. Allow the plates to sit in the hood for 30 min. to evaporate any residual test substance. Return the plates to the incubator for an additional 24-48 hrs. incubation.

7. After 2-3 days total incubation, colonies are counted with the aid of an automatic colony counter. Sample precipitate may cause difficulty in counting plates by machine. In this case and in similar cases, individual plates may be counted manually. The decision of whether or not to use the colony counter in individual cases is left to the discretion of a trained technician.

### 9.0 Data Acquisition and Analysis:

Revertant colonies are counted using a calibrated Artek Autocount colony counter or hand counted and data are properly recorded on data sheets. Before counting the revertant plates, confluent growth of the background bacterial lawn is verified visually, and in cases of poor growth, microscopic examination may be performed to identify a toxic response.

The mutagenic activity of each dose level of test substance tested in a specific tester strain is evaluated by comparing the mean (average of triplicate plates) number of revertant colonies to the mean number of colonies appearing on the solvent control plates.

A test substance is regarded as a positive mutagen if it produces a significant dose dependent increase in the mean number of revertants and if one or more of the doses produces a level 2.0 or more (3.0 for strains TA1535, TA1537, and TA1538) higher than the mean solvent control number. A test substance is considered weakly positive if it produces either a significant dose dependent increase or if one or more of the doses exhibits a significant increase in mean revertants.

Experimental data are further analyzed with the "Salmonella Assay Analysis" software (prepared by Integrated Laboratory Systems and sponsored by the Environmental Monitoring Systems Laboratory, EPA and the U.S. Army Biomedical R&D Laboratory), however, the study director makes the final interpretation.

### 5.0 Final Report:

The original and four copies of the final report will be submitted to the sponsor.

## ILS: Salmonella Typhimurium Microsome Reverse Mutation Assay

---

The final report will include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results.

### 6.0 Records to be Maintained:

Raw data sheets, statistical printouts, data diskettes, and a copy of the final report will be archived at ILS, 800-12 Capitola Dr., Durham, NC 27713 at the completion of the study until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. After this time all items will be transferred to the sponsor for archiving.

### 7.0 GLP Compliance:

This study will be conducted according to Good Laboratory Practice regulations as described by the EPA in 40 CFR Part 792. Alterations of the study protocol (if any) will be addressed in separate amendments/deviations and maintained with the protocol.

### 8.0 Quality Assurance:

Quality assurance inspections of ongoing studies will be conducted at intervals adequate to assure the integrity of the study. At least one critical phase will be inspected for each study. An audit of the final report will be conducted to verify that reported values are supported by the data and that no conditions are present that might affect the outcome of the study.

### 9.0 References:

1. Haworth, S., T. Lawlor, K. Mortelmans, W. Speck, and E. Zeiger (1983). Salmonella mutagenicity test results for 250 chemicals. Environ. Mutagen. Suppl. 1: 3-142.
2. Ames, B.N. (1971). Detection of chemical mutagens with enteric bacteria. In: Chemical Mutagens, A. Hollaender (ed.), Vol. 1, pp. 267-282. New York, Plenum Press.
3. Ames, B.N., F.D. Lee, and W.E. Durston (1973). An improved bacteria test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. USA 70(3): 782-786.
4. McCann, J., E. Choi, E. Yamasaki, and B.N. Ames (1975). Detection of carcinogens as

IIS: Salmonella Typhimurium Microsome Reverse Mutation Assay

mutagens in the Salmonella microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72(12): 5135-5139.

5. Ames, B.N., J. McCann, and E. Yamasaki (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res. 31: 347-374.

6. Maron, D.M. and B.N. Ames (1983). Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113: 173-215.

**APPROVALS**

Study Director: Paul Goshen Date: 1/31/96

Sponsor: LeRoy W. Mitten Date: 2/2/96

INTEGRATED LABORATORY SYSTEMS  
PROTOCOL AMENDMENT

SPONSOR CODE: A1

PROTOCOL AMENDMENT NO.: 1

ILS PROJECT NO.: A073-001

CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: Salmonella Typhimurium/Microsome Reverse Mutation Assay

**Change Made:**

Based on the data collected from the initial toxicity test, the following five doses are selected for the mutation assay in both the absence and presence of S9 activation:

100, 75, 50, 25, and 10% FE-13, 100% nitrogen, and 100% air/plate.

**Section Changed:**

8.3 Dose Range Selection

**Reason:**

Protocol requirement.

Submitted by:

  
Study Director

  
Date

**USACHPPM**

**U.S. Army Center for Health Promotion  
and Preventive Medicine**



**Mutagenicity Testing of  
FE-13**

*Readiness Thru Health*

## Executive Summary

Since the fire extinguishant, Halon 1301 poses an atmospheric ozone depletion potential and environmental regulations no longer allow its production, a suitable replacement must be found. One product currently under examination as a replacement for Halon 1301 is FE-13. (Freon 23; trifluoromethane;  $\text{CHF}_3$ ) is a halogenated hydrocarbon considered to be chemically inert although it can release fluoride when exposed to flame or red-hot metal. The median lethal concentration ( $\text{LC}_{50}$ ) of FE-13, based on a 4-hour exposure, is  $>650,000$  ppm. A Toxicity Profile developed for The Army Program Executive Office, Armored Systems Modernization by the Toxicology Division, AEHA in 1994 indicated that no effects were observed for FE-13 in 90-day exposure regimes at 10,000 ppm (1%). The effective extinguishant concentration of FE-13 is 12%. The no observable adverse effects level (NOAEL), based on cardiac sensitization, for FE-13 is greater than 30%. The Toxicity Profile indicated that further testing was necessary to determine developmental, reproductive and mutagenicity potential.

Genotoxic testing is an important component of a toxicological profile. Compounds which induce alterations in nucleic acids and associated components are considered to be genotoxic. Mutagenic testing is a specific type of genotoxic testing. Mutagens can induce types of stable changes in the nucleotide sequence of genes, the chromosome structure, or the chromosome number. These types of genetic events are responsible for a large proportion of human genetic diseases and congenital defects.

The compound FE-13 was tested for its mutagenic potential using four separate test systems. Each test system examined a specific mutagenic component. These test procedures included both *in vivo* and *in vitro* assays.

The AS52/GPT mammalian mutagenesis assay examines a compound's ability to induce gene mutations in the genes which code for the enzyme guanine phosphoribosyl-transferase (*gpt*) of cultured AS52 Chinese hamster ovary cells<sup>1</sup>. The addition of the metabolic activator, S9, allows the identification of promutagens. This test procedure is capable of identifying agents which cause small and large deletion mutations as well as point mutations. Also, this assay can demonstrate the cytotoxicity of the compound by comparing the cloning efficiency of treated cultures with that of nontreated cultures. Cultures were exposed to air concentrations of FE-13 for five hours at 37°C. Concentrations of FE-13, with and without the S9 activator, were 10, 25, 50, 75, and 100%. Some cultures were exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

The *Salmonella typhimurium* / microsome reverse mutation assay (Ames test), developed by Bruce Ames, is an elegant assay for the determination of mutagenicity<sup>2</sup>. This assay employs bacterial strains that are unable to manufacture histidine and is capable of detecting both base pair substitutions and frameshift mutations. The metabolic activator, S9, is used in this test to identify promutagens. The concentrations of FE-13 used in this test procedure were 10, 50, and 100% per plate. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

*In vitro* Chromosome aberrations can also be examined using Chinese hamster ovary (CHO) cells<sup>1</sup>. This assay is sensitive to clastogenic activity of a variety of chemicals. The

detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage. The S9 fraction of rat liver homogenate is also used in this test system to identify promutagens. Toxicity of FE-13 was examined in cultures, with and without S9, using concentrations of 5, 10, 25, 50, 75, and 100% and a four hour exposure period. One group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. This procedure examined average generation time, mitotic index, polyploid index, and cell density. Clastogenic activity was evaluated using concentrations of 50, 60, 70, 80, 90, and 100% in the presence or absence of the S9 activator. As with the toxicity portion of this study, one group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. A continuous exposure protocol in the absence of S9 was not practical due to the potential adverse effects of oxygen deprivation. FE-13 was found to induce a significant level of clastogenic damage in concentrations of 80% and above in the absence of metabolic activation. Control (air only) cultures containing S9 displayed a 2% (not statistically significant) increase in cellular damage while nonactivated cultures displayed no damage. This difference in baseline activity may have accounted for the nonsignificant increase in cellular damage with the S9 activator although the level of damage from exposure was identical with and without the S9 activator. Cells exposed to 100% nitrogen also displayed the same level of damage. Damage, therefore, is probably due to a decreased oxygen level rather than the activity of FE-13.

The mouse bone micronucleus assay is an *in vivo* test system which can determine the ability of a compound to induce micronuclei formation in immature erythrocytes of male and female mice<sup>3</sup>. Micronuclei are formed when chromosomes lag or fragment during cell division. The B6C3F1 strain of mouse was used in this study as this strain appears to be exquisitely sensitive to micronucleus induction. This assay is the most reliable method for evaluating the potential of a compound to induce clastogenic or aneugenic damage. FE-13 was assayed using concentrations of 13%, 26%, 50%. Control animals were exposed to 100% air as well as an oxygen poor environment of 50% air and 50% nitrogen. FE-13 did not induce a significant level of mutagenicity.

The results of the above tests indicate that FE-13 does not induce a mutagenic effect at dosage levels tested and, from a mutagenicity standpoint, it appears to be a suitable replacement for Halon 1301. Further genotoxicity testing of this material is not indicated at this time.

## References

1. Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolf, and J.S. Wassom. (1981). Mammalian *in vivo* and *in vitro* cytogenic assays: A report of the U.S.E.P.A. Gen-Tox Program. *Mutation Res.* 87:143-188.
2. Ames, B.N., J. McCann, and E. Yamasaki. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella* mammalian microsomal mutagenicity chromosome. *Mutation Res.* 31:347-364.
3. Heddle, J.A., M. Hite, B. Kirkhart, K. Mavroumin, J.T. MacGregor, G.W. Newell, and M.F. Salamone. (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S.E.P.A. Gene-Tox Program. *Mutation Res.* 123:61-118.



WILFRED C. McCAIN, Ph.D.  
Toxicologist  
Health Effects Research Program  
U.S. Army Center for Health Promotion and  
Preventive Medicine



JOSEPH A. MACKO, JR  
Liaison Officer  
Army Acquisition Pollution Prevention  
Support Office  
U.S. Army Materiel Command

APPROVED:



LERoy W. METKER  
Program Manager  
Toxicity Evaluation Program  
U.S. Army Center for Health Promotion and Preventive Medicine