Final Report

Bacterial Reverse Mutation Screening Assay using *Salmonella typhimurium*

Test Article:	EB (Lot # 054K3681)
Date Initiated:	February 21, 2006
Date Completed:	March 14, 2006
Protocol:	A70S-2006
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2. Objective

This Bacterial Reverse Mutation Screening Assay was performed to evaluate the ability of this test article to induce a mutagenic response in two strains of *Salmonella typhimurium* (TA98 and TA1537).

3. Test Article and Vehicle Description

Test article characteristics:	Test article was received as a dark red, almost purple powder in a clear vial labeled: "Cat E8751, Lot: 054K3681, 50 mg, Ethidium Bromide, For Research Use Only." The protocol indicated that this test article expires 6/15/2006.
Storage conditions:	The test article was stored at -24 ºC to -21 ºC, protected from light.
Vehicle (and lot number):	Dimethyl sulfoxide (Lot: 05754KD, Exp: 9/07).
Justification for vehicle choice:	Sponsor indicated vehicle to be utilized.
Description of test article when mixed with vehicle:	Dark purple solution.

4. Summary

The results of this Bacterial Reverse Mutation Screening Assay indicate that under the experimental conditions, this test article was mutagenic for both tester strains, with 10% S9 metabolic activation, and strain TA1537 without S9 metabolic activation.

Protocol A70S-2006 is based on Organisation for Economic Co-operation and Development (OECD) and International Conference on Harmonisation (ICH) testing guidelines.

5. Materials and Methods

5.1. Test System Description

The Salmonella strains used were histidine-dependent. Revertants were identified as colonies that grew in low levels of histidine. Frameshift substitution defects were represented to identify mutagens. Additional genetic markers enhanced sensitivity of the strains to certain types of mutagens.

The DNA repair mutation (*uvrB*) eliminates excision repair, a repair pathway for DNA damage from UV light and certain chemical mutagens. The *uvrB* mutation, present in both strains, was indicated by sensitivity to UV light. The *rfa* mutation changes the properties of the bacterial cell wall, increasing permeability of cells to certain types of chemicals. The *rfa* mutation, present in both strains was indicated by sensitivity to crystal violet.

The R factor plasmid (pKM101) present in strain TA98 makes it more responsive to a variety of mutagens. The plasmid carries an ampicillin resistance gene; therefore ampicillin resistance indicated that the strain retain the plasmid.

CHARACTERISTICS OF TESTER STRAINS						
Tester Strain	**Gene Affected**	**DNA Repair**	**LPS**	**Biotin Requirement**	**Plasmids**	**Mutational Event**
TA98	*hisD*	*uvrB*	*rfa*	bio-	pKM101	frameshift
TA1537	*hisC*	*uvrB*	*rfa*	bio-	-	frameshift

5.2. Test System Justification

The two strains of bacteria used in this assay are among those recommended by OECD 471 for use in the Ames test. These two strains of *S. typhimurium* have been shown to be reliably and reproducibly responsive between laboratories.

5.3. Source and Storage of Test System

The Salmonella strains used in this study were obtained from Molecular Toxicology, Inc. Cells are maintained as frozen stocks (-85 ºC ± 4 ºC).

5.4. Identification of Test System

Strains TA98 and TA1537 were identified by having certain characteristics (see above). The strains also yielded spontaneous revertant colony plate counts within the frequency ranges stated in the historical control data.

5.5. Preparation of Overnight Cultures

Frozen stock cultures (stored at -85 °C ± 4 °C) were grown overnight at 37 ± 2 °C, with shaking, in nutrient broth until a cell density of 1 x 10^9 to 2 x 10^9 cells/ml was obtained (determined by optical density). Cells were refrigerated until use and maintained at room temperature during the test.

5.6. Control of Bias

In order to control bias on the day of test system treatment, all test article doses, as well as controls, were plated against cells obtained from a single flask for each strain.

5.7. Metabolic Activation

5.7.1. S9 Fraction

Aroclor™ 1254-induced male Sprague Dawley rat liver S9 (500 mg/kg i.p.), was purchased from a commercial supplier (Molecular Toxicology, Inc. Boone, NC). Lot 1938 was used, which contains 40.8 mg/ml protein. This lot has demonstrated the ability to activate ethidium bromide, cyclophosphamide, benzo(a)pyrene, and 2-aminoanthracene into mutagenic intermediates, and has demonstrated P450 substrate activation in the ethoxyresorufin-0-deethylase, pentoxyresorufin-0-dealkylase, benzylresorufin-0-dealkylase and methoxyresorufin-0-dealkylase assays.

5.7.2. S9/Cofactor Mix

The S9/Cofactor Mix was prepared immediately before the test and contained: 10% S9, magnesium chloride, potassium chloride, D-glucose-6-phosphate, and nicotinamide adenine dinucleotide phosphate, in a sodium phosphate buffer. It was kept on ice during the experiment.

5.7.3. Buffer

When S9 mix was not used in the test, phosphate buffered saline (PBS) was used in its place.

5.8. Tester Strain Media

5.8.1. Nutrient Broth

The broth used for the overnight cultures consisted of 2.5% Oxoid Nutrient Broth #2.

5.8.2. Vogel-Bonner Plates

Minimal glucose agar plates (1.5% agar supplemented with 2.0% glucose and 2.0% Vogel-Bonner buffer) were purchased from a commercial supplier (Moltox, Boone, NC).

5.8.3. Top Agar

Top agar was prepared with 0.6% agar and 0.6% NaCl supplemented with histidine (0.5 mM) and biotin (0.5 mM). For the assay, 2.0 ml supplemented top agar was used.

5.9. Definitive Assay

Concentrations of test article prepared:	0, 1, 5, 10, 50, 100, 250, 500, 1000, 2500 and 5000 µg/ml.
Volume of each concentration plated:	0.1 ml per plate.
Doses tested:	0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 250 and 500 µg/plate.
Justification for doses tested:	Sponsor requested these concentrations be tested.
Number of plates per dose:	Duplicate plates were used for each dose.
Microbiological contamination:	An aliquot of the top concentration was plated to test for microbiological contamination. None was evident.

The following was added to each sterile culture tube containing 2.0 ml top agar: 0.1 ml of overnight cell culture (TA98 or TA1537), 0.1 ml of each test article concentration or control chemical, and either 0.5 ml of S9/Cofactor mix or 0.5 ml of phosphate buffered saline.

The contents of each tube were vortexed, poured onto Vogel-Bonner media plates, and evenly distributed. The agar on the test plates was allowed to harden. The plates were inverted and incubated at 37 ºC ± 2 ºC for 48 hours ± 4 hours prior to scoring.

Was test article precipitation observed? If yes, at what doses?	No test article precipitation was observed.
Was toxicity observed? If yes, at what doses?	Toxicity was observed at the 500 µg/plate dose for strain TA98, without S9 metabolic activation.

5.10. Counting

5.10.1. Automatic Colony Counting

A New Brunswick Biotran III automatic colony counter was used for counting revertant colonies. The control chemical plates were counted before the test article plates for each strain to ensure that the strain was functioning properly. Before counting, each plate was scanned for contamination, test article precipitation, toxicity and any foreign material. Toxicity is suggested by the absence of a confluent bacterial lawn, the presence of pinpoint colonies, and/or a substantial decrease or lack of revertant colonies. Each plate was counted 3 times on the automatic colony counter, rotating the plate one third each time. The median count was recorded.

5.10.2. Hand Counting

Hand counting was not required.

5.11. Criteria for a Valid Assay

The study will be considered valid if the following criteria are met.

- Both tester strains demonstrated the presence of the *uvrB* mutation by exhibiting sensitivity to UV light.
- Both tester strains demonstrated the presence of the *rfa* wall mutation by exhibiting sensitivity to crystal violet.
- Tester strain TA98 demonstrated the presence of the pKM101 plasmid by exhibiting resistance to ampicillin.
- Each tester strain demonstrated a characteristic number of spontaneous revertant colonies. A "characteristic number" is defined as the average number of colonies across plates being within the historical range, or within the published historical range.
- Each tester strain exhibited at least a three-fold increase in average mutagen-induced revertant colonies when plated with positive control chemicals.

5.12. Statistical Analysis of the Data

Only when an assay is valid, and only when any test article treatment group demonstrates an increase in average number of revertant colonies relative to the negative control, will data be subjected to statistical analyses.

The average of each set of duplicate plates was determined. JMP software's regression analysis (v5) was used to determine if a dose-related increase occurred ($p < 0.025$). A statistically significant dose-related increase was observed for both strains TA98 ($p = 0.0015$) and TA1537 ($p < 0.0001$) with S9 metabolic activation. This statistically significant dose-related increase was also observed for strain TA1537 without S9 metabolic activation ($p < 0.0001$).

5.13. Determining a Positive Response

The test article will be considered positive if the assay is valid, and if the following conditions are met, taking into account biological relevance:

- One test article dose exceeds three times the background average (two times for strain TA1537) either with or without metabolic activation, or there is a dose-related increase over the range tested ($p < 0.025$).
- If the background average is below six colonies, the average number of revertants for the test article must exceed 20 colonies/plate.

A positive result indicates that the test article induces mutations in *Salmonella typhimurium* cells.

A test article for which the results do not meet the above criteria will be considered non-mutagenic in this test. Negative results indicate that, under the test conditions, the test article does not produce mutations in *Salmonella typhimurium* cells.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test article. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

6. Historical Control Data

Average Number of Colonies per Plate ± Standard Deviation (Range Indicated Below)		
	Salmonella typhimurium strains	
Agent	**TA98**	**TA1537**
Top Agar + His	11 ± 3 (7 - 16)	6 ± 2 (2 - 10)
DMSO	11 ± 5 (4 - 22)	5 ± 2 (1 - 9)
Positive Control (μg/plate)	**2NF (1)**	**9AA (50)**
	224 ± 65 (132 - 313)	93 ± 49 (40 - 225)
Top Agar + 10% S9	19 ± 5 (9 - 28)	4 ± 2 (1 - 7)
DMSO + 10% S9	18 ± 4 (13 - 26)	5 ± 2 (1 - 8)
Positive Control + 10% S9 (μg/plate)	**BP (3)**	**2AAn (5)**
	281 ± 25 (229 - 342)	144 ± 21 (96 - 171)

Historical data determined from six Ames tests, February through March 2006.

7. Legend

His:	histidine	
S9:	S9 metabolic activation	
DMSO:	dimethyl sulfoxide	CAS#: 67-68-5
2NF:	2-nitrofluorene	CAS#: 607-57-8
NaN_3:	sodium azide	CAS#: 26628-22-8
BP:	benzo(a)pyrene	CAS#: 50-32-8
2AAn:	2-aminoanthracene	CAS#: 613-13-8

8. Discussion of Results

Strain TA98 with S9 metabolic activation met both criteria for a positive response as follows: the average number of revertant colonies for the 1 μg/plate dose and higher, was at least 3-fold higher than the average number of revertant colonies of the corresponding vehicle controls; and a statistically significant dose-related increase was observed.

Strain TA1537 both with and without S9 metabolic activation met the criteria for a positive response as follows: the background average was below six colonies and more than 20 colonies per plate were observed at the 25 μg/plate dose with S9 metabolic activation and at the 500 μg/plate dose without S9 metabolic activation; and a statistically significant dose-related increase was observed.

9. Conclusions

The results of this assay indicate that under the experimental conditions, this test article was mutagenic for strains TA98 and TA1537, with 10% S9 metabolic activation, and for strain TA1537, without S9 metabolic activation.

10. Records Maintained

All records regarding the study, including correspondence between the sponsor and Litron, the protocol, amendments to the protocol, data sheets, environmental and equipment information, training records, historical data, a copy of the final report, and all other raw data and applicable information, will be maintained at Litron for five years following completion of the study. Electronic copies of records will be stored off-site (315 Root Rd., Brockport, NY 14420) in addition to storage at Litron Laboratories.

11. References

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

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- Maron, D., J. Katzenellenbogen, and B. Ames (1981) Compatibility of organic vehicles with the Salmonella/microsome test. Mutation Res. 88, 343-350 Maron, D., J. Katzenellenbogen, and B. Ames (1981) Mutation Res. 88, 343-350.

- McKee, R., J. Tometsko, and A. Tometsko (1979) Chemicals which revert all commonly used *Salmonella typhimurium* tester strains. Mutation Res. 67, 183-187.

- Mortelmans K. and Ziegler E. (2000) Mutatation Res. 455, 29-60.

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

- International Conference on Harmonisation (ICH) Tripartite Guidelines: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A, adopted July 19, 1995.

- International Conference on Harmonisation (ICH) Tripartite Guidelines: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B, adopted July 16, 1997.

- Organisation for Economic Cooperation and Development (OECD) Section 4 of the OECD Guidelines for the Testing of Chemicals: Bacteria Reverse Mutation Test, Guideline 471, adopted July 21, 1997.

Table 1: Control Data

Date plated: March 1, 2006
Date counted: March 3, 2006

Raw Data (Number of Colonies per Plate)

	***Salmonella typhimurium* Strains**			
	TA98		**TA1537**	
Top Agar + His	8	10	C	5
DMSO	10	11	2	1
Positive Control	2NF (1 μg/plate)		9AA (50 μg/plate)	
	172	187	203	190
Top Agar + S9	15	15	6	8
DMSO +S9	13	15	4	7
Positive Control + S9	BP (3 μg/plate)		2AAn (5 μg/plate)	
	242	250	157	152

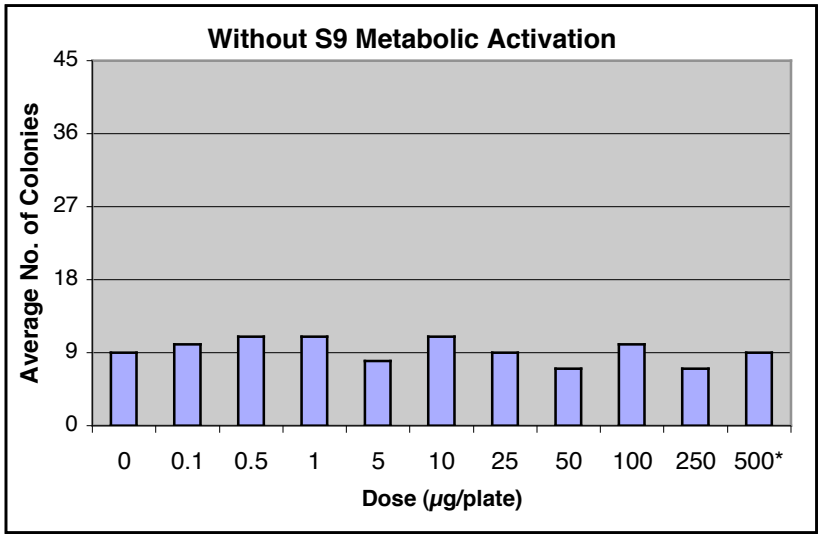
Average Number of Colonies per Plate

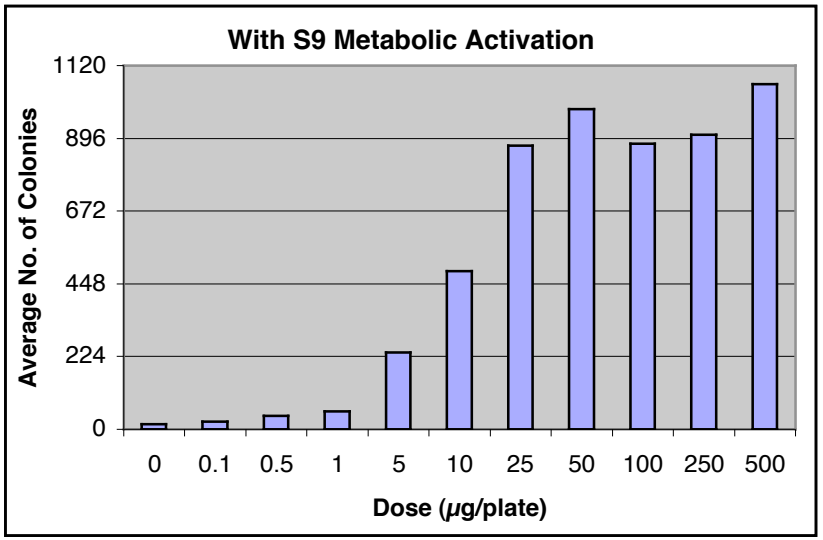
	***Salmonella typhimurium* Strains**	
	TA98	**TA1537**
Top Agar + His	9	5
DMSO	11	2
Positive Control	2NF (1 μg/plate)	9AA (50 μg/plate)
	180	197
Top Agar + S9	15	7
DMSO +S9	14	6
Positive Control + S9	BP (3 μg/plate)	2AAn (5 μg/plate)
	246	155

C = Contamination

Figure and Table 2: Strain TA98

Date plated: March 1, 2006
Date counted: March 3, 2006





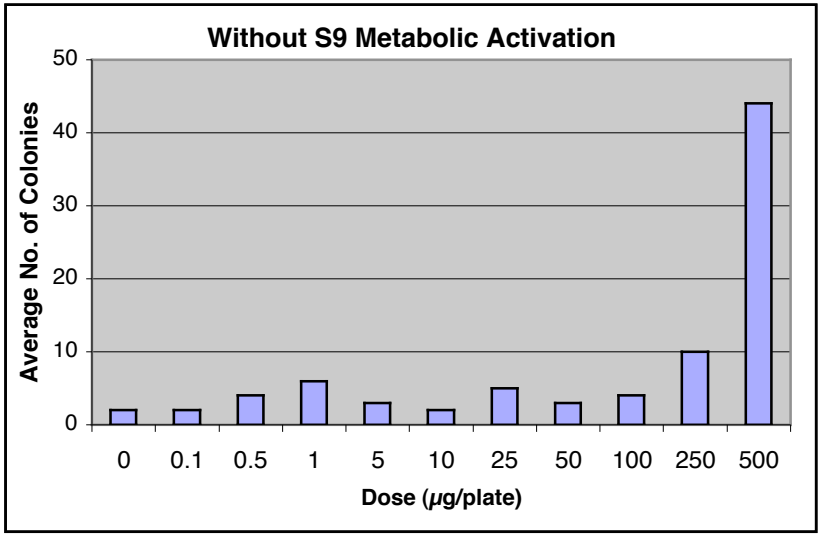
Dose (μg/plate)	Number of Colonies		Averages
0	8	9	**9**
0.1	9	10	**10**
0.5	11	10	**11**
1	11	10	**11**
5	9	7	**8**
10	11	11	**11**
25	11	6	**9**
50	7	6	**7**
100	6	13	**10**
250	5	9	**7**
500*	4	13	**9**

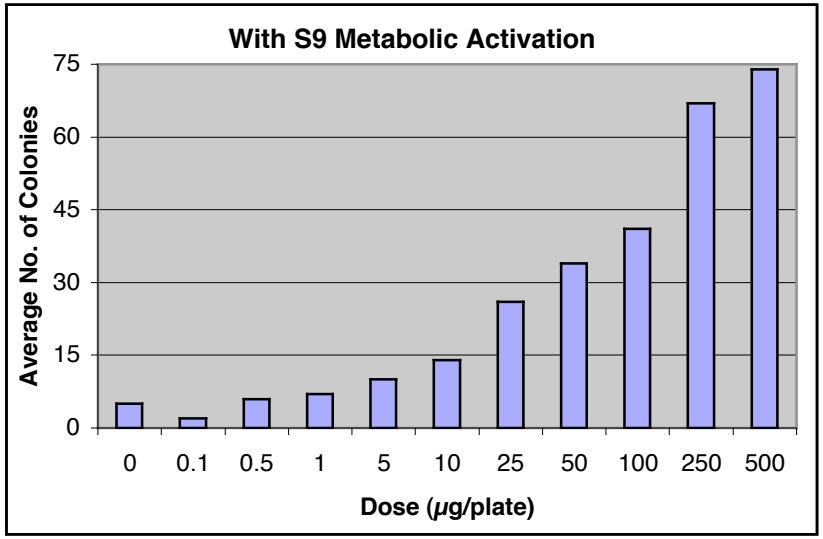
Dose (μg/plate)	Number of Colonies		Averages
0 + S9	15	16	**16**
0.1 + S9	18	30	**24**
0.5 + S9	41	40	**41**
1 + S9	61	50	**56**
5 + S9	272	200	**236**
10 + S9	541	435	**488**
25 + S9	882	866	**874**
50 + S9	1,020	949	**985**
100 + S9	1,001	758	**880**
250 + S9	1,157	657	**907**
500 + S9	1,067	1,059	**1,063**

* = Toxicity

Figure and Table 3: Strain TA1537

Date plated: March 1, 2006
Date counted: March 3, 2006





Dose (μg/plate)	Number of Colonies		Averages
0	3	1	**2**
0.1	2	2	**2**
0.5	3	4	**4**
1	4	7	**6**
5	4	1	**3**
10	2	2	**2**
25	2	7	**5**
50	2	4	**3**
100	3	5	**4**
250	9	10	**10**
500	41	46	**44**

Dose (μg/plate)	Number of Colonies		Averages
0 + S9	5	5	**5**
0.1 + S9	1	3	**2**
0.5 + S9	8	3	**6**
1 + S9	5	9	**7**
5 + S9	9	11	**10**
10 + S9	15	12	**14**
25 + S9	26	25	**26**
50 + S9	22	45	**34**
100 + S9	46	35	**41**
250 + S9	72	62	**67**
500 + S9	90	58	**74**