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FINAL REPORT

SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY: AMES TEST

PROTOCOL NO. 200501303-01

LABORATORY NO. 281931

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STUDY DIRECTOR GLP CERTIFICATION

[X] USFDA (21 CFR PART 58) [ ] USEPA (40 CFR PART 160)

SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY: AMES TEST

I CERTIFY THAT THE TEST WAS CONDUCTED IN ACCORDANCE

WITH THE USFDA OR USEPA REGULATIONS AS NOTED ABOVE.

LABORATORY NO. 281931

STUDY DIRECTOR: _____ DATE: 3/[illegible]/2005

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QAU AUDIT STATEMENT

[X] USFDA (21 CFR PART 58) [ ] USEPA (40 CFR PART 160)

SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY: AMES TEST

Study Director: Chad Summers, A.S.

Final Report Dated: 26 Jan 2005

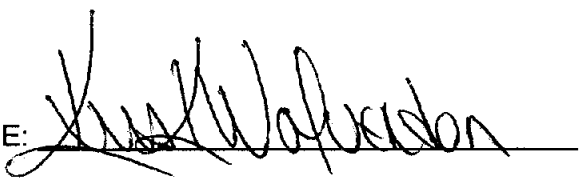
1. The test was conducted in accordance with the USFDA or USEPA Regulations as noted above. All laboratory results pertaining to this study are recorded in Nelson Laboratories' Data File Number 281931.

2. In accordance with the Good Laboratory Practice Regulations, the Test Procedure phase(s) of this study was inspected by the Quality Assurance Unit on: 21 Jan 2005. The findings of the inspection(s) were reported to Management and to the Study Director on: 24 Jan 2005.

3. The Quality Assurance Unit has reviewed this report and has determined that the methods and standard operating procedures are accurately described, and that the reported results accurately reflect the raw data.

4. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study:

Michelle Lee
Chad Summers
Heidi Waldron
Dr. Jerry Nelson
Jeff Hills

QUALITY ASSURANCE:  DATE: 31 Jan 2005

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SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY: AMES TEST

LABORATORY NUMBER:	281931
PROTOCOL NUMBER:	200501303-01
SAMPLE SOURCE:	Optigenex, Inc.
SAMPLE IDENTIFICATION:	C-MED-100® AKA AC-11™ Lot #0929053359
DEVIATIONS:	None
DATA ARCHIVE LOCATION:	Sequentially by lab number
PROTOCOL APPROVAL DATE:	20 Jan 2005
SAMPLE RECEIVED DATE:	12 Jan 2005
LAB PHASE START DATE:	20 Jan 2005
LAB PHASE COMPLETION DATE:	25 Jan 2005
REPORT ISSUE DATE:	26 Jan 2005
TOTAL NUMBER OF PAGES:	16

REFERENCES:

Ames, B. N., McCann, J., and Yamasaki, E. 1975. Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. Mutation Res., 31:347-364.

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

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ANSI/AAMI/ISO 10993-3: 2003. Biological Evaluation of Medical Devices-Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity. ANSI/AAMI/ISO.

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INTRODUCTION:

The *Salmonella typhimurium* reverse mutation (Ames) test employs several strains of *S. typhimurium* which require the amino acid histidine for growth to detect point mutations. The test detects mutations which cause the bacterial strains to revert to bacteria which are capable of synthesizing histidine. These revertants are detected by their ability to grow in the absence of

histidine. These strains have been selected based on their sensitivity to mutation because of: 1) an increased cell wall permeability, due to mutation which causes partial loss of the lipopolysaccharide barrier that coats the bacterial surface and results in increased permeability to large molecules (rfa mutation); 2) a mutation in the bacterial cell system to excise and repair defects in the DNA, resulting in the inability to repair damaged or mutated sections (uvrB mutation); and 3) R-factor plasmids (some strains) and a multicopy plasmid (some strains) which contain error-prone DNA repair systems. The tester strains are selected to detect various types of mutagens. The tester strains employed were TA97A, TA98, TA100, TA102 and TA1535.

The *Salmonella typhimurium* reverse mutation (Ames) tests are performed using the plate incorporation method and the spot test method. The plate incorporation method involves mixing the test substance and the test organism together in a soft agar solution, which contains only small amounts of histidine. The spot test uses the same soft agar solution without the test substance. The test substance is then added as a spot on top of the hardened agar mixture. The histidine in the soft agar solution permits the inoculated test organism to undergo a limited number of divisions, but is insufficient to permit normal growth. If the strain undergoes a reverse mutation, (spontaneous or induced by the test substance or a positive control material), the organism no longer requires histidine to grow and can produce a visible colony or revertant.

The tests were performed both with and without metabolic activation using the S-9 activation system. The S-9 activation system is designed to simulate mammalian liver enzyme systems and is used to detect substances which undergo metabolic activation from non-mutagenic forms.

PROCEDURE:

Broth Culture Preparation: Commercial culture discs were used to inoculate nutrient broth for testing. The cultures were incubated at 37 ± 2°C for 10-14 hours on an orbital shaker until when measured spectrophotometrically at 660 nm, an absorbance reading of approximately 1.0 to 2.0 was obtained. Validation data of the cultures showed absorbance readings in the above range resulted in concentrations of approximately 10^9 CFU/mL.

Strain Genotype Verification: The strains used for the test were checked for presence of appropriate strain genotype characteristics. These tests included verification of the following:

- Presence of uvrB mutation
- Presence or absence of R-factor plasmid
- Presence of rfa mutation
- Requirement for histidine

The uvrB mutation was verified by demonstrating UV sensitivity (lack of repair system). The R-factor was checked by determining sensitivity or resistance to ampicillin (0.08% in 0.02 NaOH). The presence of the rfa mutation was verified by demonstrating sensitivity to crystal violet (0.1% in water) on nutrient agar plates. The histidine requirement was assured by plating onto minimal glucose agar plates spread with 0.1 mL of 0.5 mM biotin and both with and without 0.1 mL of 0.1 M histidine.

Sample Preparation: Prior to testing the sample was diluted in purified water, USP to a 0.5% concentration.

Metabolic Activation System: The S-9 activation system was used to screen for the presence of mutagens from byproducts of the test sample. Rat liver S-9 homogenate was obtained from Molecular Toxicology, Inc. The homogenate was kept frozen at ≤-60°C upon receipt. Plates requiring activation contained approximately 20 µL S-9 per plate. When working with soft agar the plates did not exceed 47°C.

Top Agar Preparation: Aliquots of top agar were melted and maintained at 45 ± 2°C. Each 100 mL aliquot of top agar was fortified with approximately 10 mL of 0.5 mM biotin and 0.5 mM histidine prior to use.

Plate Incorporation Tests: Two mL aliquots the top agar were transferred to sterile tubes and 0.1 mL of the appropriate test organisms was added. Three tubes of top agar mixture with the test organism were plated on minimal glucose agar plates without the addition of either test or chemical control material and used to establish the spontaneous reversion rate. The test article was tested by adding 0.1 mL of the sample to three tubes of top agar mixture then plating on minimal glucose plates. The sample was tested with and without the S-9 activation system. The plates were incubated for growth of the organisms at 37 ± 2°C for 48-72 hours.

Spot Tests: The test material was also tested in spot tests on plates with and without the S-9 activation system. Two mL aliquots of the top agar mixture and 0.1 mL of the appropriate test organism was added to minimal glucose agar plates. The plates were allowed to harden then 10 µL of the test material was added as a spots on the surface of the plate. The plates were incubated for growth of the organisms at 37 ± 2°C for 48-72 hours.

Chemical Control Materials: The following chemical controls were used Sodium Azide, Mitomycin-C, 4-nitro-0-phenylene-diamine (NPD), and 2 aminofluorene (2AF). The chemical controls were tested using the plate incorporation and spot test with and without the S-9 activation system.

Acceptance Criteria: The criteria for acceptance of the test and criteria for determination of a mutagen are listed below.

1) Tested in the presence and the absence of liver microsomal enzymes preparation.
2) Tested strains for genotype verification and achieved the appropriate responses.
3) All chemical controls included in the test gave the appropriate responses.

Criteria for a Mutagen: 1) A two-fold increase over the spontaneous reversion rate (percent of control >200%).

2) Demonstration of a dose-response curve when dilutions are tested.

Criteria for a Non-Mutagen: 1) A less than two-fold increase over spontaneous reversion rate (percent of control <200%).

2) No dose response curve when dilutions are tested.

RESULTS/CONCLUSION:

The test results are summarized in Tables 1-7. The results are calculated using a validated computer program. Manual calculations may differ slightly due to rounding. Tables 1-5 contain the results for the plate incorporation tests. They include the spontaneous revertant control, test substance and chemical control plates. Table 6 contains the results for the spot tests recorded as + (positive) or - (negative). Table 7 contains the results for the genotype verification. All five tester strain cultures showed the appropriate results in the genotype verification assay.

SUMMARY:

None of the 5 tester strains produced two-fold increases in the number of spontaneous revertants. The spot tests showed no zone of increased reversion or of inhibition. In summary, the sample (0.5%) tested against the five strains did not meet the criteria for a potential mutagen.

STATEMENT OF UNCERTAINTY:

This is a pass/fail test based on the visual inspection of the interaction of only two elements. Results are expressed as greater than, or less than, a 200% increase in certain visible aspects. The uncertainty data attributable to other measurable parameters of the test result in a combined standard uncertainty of 0.046 RSD and an expanded uncertainty, at a 95% confidence level, of 0.093 RSD. Other than these values, the test data is not conducive to statistical analysis.

Chad Summers, A.S.
Study Director

Study Completion Date

CJS/mjm

TABLE 1. TA97A Results
(Number of Revertants)

Results Without Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	78	106	104	96	
Test Sample:	94	119	86	100	104
Sodium Azide:	111	114	104	110	114 (-)
NPD:	565	570	643	593	617 (+)
2AF	110	131	92	111	116 (-)
Results With S-9 Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	135	102	110	116	
Test Sample:	104	111	102	106	91
Sodium Azide:	114	106	119	113	98 (-)
NPD:	532	320	394	415	359 (+)
2AF:	651	692	664	669	578 (+)

Note: Percent of control results greater than 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls are included as ± in the parentheses ( ).

TABLE 2. TA98 Results
(Number of Revertants)

Results Without Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	30	24	20	25	
Test Sample:	27	17	29	24	99
Sodium Azide:	24	24	17	22	88 (-)
NPD:	1055	1125	1137	1106	4482 (+)
2AF	32	26	33	30	123 (-)
Results With S-9 Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	21	35	30	29	
Test Sample:	21	25	24	23	81
Sodium Azide:	23	33	29	28	99 (-)
NPD:	1277	1250	1252	1260	4394 (+)
2AF:	1828	1788	1817	1811	6317 (+)

Note: Percent of control results greater than 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls are included as ± in the parentheses ( ).

TABLE 3. TA100 Results
(Number of Revertants)

Results Without Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	81	114	93	96	
Test Sample:	77	79	87	81	84
Sodium Azide:	269	340	303	304	317 (+)
NPD:	431	450	390	424	441 (+)
2AF	95	78	88	87	91 (-)
Results With S-9 Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	96	102	119	106	
Test Sample:	116	102	113	110	104
Sodium Azide:	222	300	301	274	260 (+)
NPD:	299	354	338	330	313 (+)
2AF:	1054	900	1008	987	934 (+)

Note: Percent of control results greater than 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls are included as ± in the parentheses ( ).

TABLE 4. TA102 Results
(Number of Revertants)

Results Without Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	195	189	187	190	
Test Sample:	200	216	233	216	114
Sodium Azide:	192	172	189	184	97 (-)
NPD:	222	226	203	217	114 (-)
Mitomycin-C:	977	988	987	984	517 (+)
Results With S-9 Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	254	256	275	262	
Test Sample:	263	261	260	261	100
Sodium Azide:	267	265	263	265	101 (-)
NPD:	270	299	252	274	105 (-)
Mitomycin-C:	1115	1171	1179	1155	441 (+)

Note: Percent of control results greater than 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls are included as ± in the parentheses ( ).

TABLE 5. TA1535 Results
(Number of Revertants)

Results Without Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	19	12	12	14	
Test Sample:	13	16	15	15	102
Sodium Azide:	262	261	288	270	1886 (+)
NPD:	16	22	16	18	126 (-)
2AF	20	12	15	16	109 (-)
Results With S-9 Activation:					
Identification:	Plate Count Results:			Average:	Percent of Control:
Spontaneous Control:	19	28	17	21	
Test Sample:	18	17	22	19	89
Sodium Azide:	153	210	205	189	888 (+)
NPD:	17	14	20	17	80 (-)
2AF:	26	34	30	30	141 (-)

Note: Percent of control results greater than 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls are included as ± in the parentheses ( ).

TABLE 6. Spot Test Results

Results Without Activation:					
Identification:	TA97a	TA98	TA100	TA102	TA1535
Test Sample:	-	-	-	-	-
Sodium Azide:	- (-)	- (-)	+ (+)	- (-)	+ (+)
NPD:	+ (+)	+ (+)	+ (+)	- (-)	- (-)
2AF:	- (-)	- (-)	- (-)		- (-)
Mitomycin-C				+ (+)	
Results With S-9 Activation:					
Identification:	TA97a	TA98	TA100	TA102	TA1535
Test Sample:	-	-	-	-	-
Sodium Azide:	- (-)	- (-)	+ (+)	- (-)	+ (+)
NPD:	+ (+)	+ (+)	+ (+)	- (-)	- (-)
2AF:	+ (+)	+ (+)	+ (+)		- (-)
Mitomycin-C				+ (+)	

Note: Results are reported as ±. The expected result for the controls are included in parentheses ( ).

TABLE 7. *Strain Verification Results*

PARAMETER	STRAINS				
	TA97A	TA98	TA100	TA102	TA1535
uvrB	+	+	+	-	+
R-factor	+	+	+	+	-
rfa	+	+	+	+	+
Histidine Requirement	+	+	+	+	+