NELSON LABORATORIES

Sponsor:
Rose Cattolico
University of Washington
P.O. Box 355640
Seattle, WA 98195

The *Salmonella typhimurium* Bacterial Reverse Mutation Assay (Ames Test), Liquids or Soluble Chemicals Final Report

Test Article:	DLE-13-180
Purchase Order:	EI582632
Laboratory Number:	853260-S01
Study Received Date:	22 Oct 2015
Test Procedure(s):	Protocol Number: 201500992 Rev 01

Summary: The *Salmonella typhimurium* reverse mutation assay (Ames test) is used to determine the potential mutagenic activity of the test article by exposing a large number of the test organism to the test article in agar plates. The agar plates are monitored for growth of revertants (organisms mutating to the wild type) which are counted and used to estimate the mutagenic potential of the test article.

The Ames test employs several histidine dependent (His-) strains of *S. typhimurium* which require the amino acid histidine for growth. The test detects mutations which cause the bacterial strains to revert to histidine independent (His+) bacteria which are capable of synthesizing histidine and can grow in the absence of histidine. The assay uses tester strains TA97a, TA98, TA100, TA102 and TA1535 which were selected to detect various types of mutagens. The test is performed both with and without metabolic activation using an 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.

All test method acceptance criteria were met. The test procedure(s) listed above were followed without deviation. Testing was performed in compliance with US FDA good manufacturing practice (GMP) regulations 21 CFR Parts 210, 211 and 820.

Results: The results are calculated using a validated computer program. Manual calculations may differ slightly due to rounding. All results greater than 300 colony forming units (CFU) are considered estimates.

The test article concentrations did not produce a two-fold or three-fold increase in the number of revertants or produce a clear dose related response in any of the 5 tester strains. The spot tests showed no zone of increased reversion or of toxicity. In summary, the test article concentrations tested against the five strains did not meet the criteria for a potential mutagen.

Brittany Todd

Study Director — Brittany Todd, M.S., RM(NRCM)

18 NOV 2015
Study Completion Date

TA97a (Number of Revertants): Percent of control results greater than (>) 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls is included as ± in the parentheses ( ).

Results Without Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	109	117	100	109	N/A
Test Article 5 µL/plate	88	101	94	94	87
Test Article 1.6 µL/plate	94	85	94	91	84
Test Article 0.5 µL/plate	96	87	85	89	82
Test Article 0.16 µL/plate	104	98	101	101	93
Test Article 0.05 µL/plate	101	92	97	97	89
NPD	407	396	476	426	392 (+)
Water Control	98	118	114	110	N/A
Results With S-9 Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	138	113	163	138	N/A
Test Article 5 µL/plate	137	120	146	134	97
Test Article 1.6 µL/plate	130	141	141	137	100
Test Article 0.5 µL/plate	149	143	138	143	104
Test Article 0.16 µL/plate	138	142	149	143	104
Test Article 0.05 µL/plate	122	135	149	135	98
2-AF	1,115	1,092	1,111	1,106	801 (+)
Water Control	149	126	133	136	N/A

TA98 (Number of Revertants): Percent of control results greater than (>) 300 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls is included as ± in the parentheses ( ).

Results Without Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	13	20	19	17	N/A
Test Article 5 µL/plate	16	19	16	17	98
Test Article 1.6 µL/plate	20	18	10	16	92
Test Article 0.5 µL/plate	17	14	18	16	94
Test Article 0.16 µL/plate	24	16	19	20	113
Test Article 0.05 µL/plate	18	24	14	19	108
NPD	592	646	677	638	3,683 (+)
Water Control	9	14	20	10	N/A
Results With S-9 Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	26	13	31	23	N/A
Test Article 5 µL/plate	25	22	12	20	84
Test Article 1.6 µL/plate	9	20	19	16	69
Test Article 0.5 µL/plate	17	29	18	21	91
Test Article 0.16 µL/plate	25	26	25	25	109
Test Article 0.05 µL/plate	26	27	22	25	107
2-AF	2,405	2,399	2,390	2,398	10,277 (+)
Water Control	21	21	13	18	N/A

TA100 (Number of Revertants): Percent of control results greater than (>) 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls is included as ± in the parentheses ( ).

Results Without Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	148	165	154	156	N/A
Test Article 5 µL/plate	175	154	154	161	103
Test Article 1.6 µL/plate	128	154	134	139	89
Test Article 0.5 µL/plate	123	115	127	122	78
Test Article 0.16 µL/plate	156	135	140	144	92
Test Article 0.05 µL/plate	131	142	142	138	89
Sodium Azide	973	960	889	941	604 (+)
Water Control	125	141	131	132	N/A
Results With S-9 Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	158	145	168	157	N/A
Test Article 5 µL/plate	148	147	146	147	94
Test Article 1.6 µL/plate	182	147	152	160	102
Test Article 0.5 µL/plate	156	162	157	158	101
Test Article 0.16 µL/plate	163	155	147	155	99
Test Article 0.05 µL/plate	152	144	145	147	94
2-AF	1,336	1,604	1,529	1,490	949 (+)
Water Control	145	164	173	161	N/A

TA102 (Number of Revertants): Percent of control results greater than (>) 200 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls is included as ± in the parentheses ( ).

Results Without Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	275	299	284	286	N/A
Test Article 5 µL/plate	284	297	299	293	103
Test Article 1.6 µL/plate	323	298	292	304	106
Test Article 0.5 µL/plate	314	304	284	301	105
Test Article 0.16 µL/plate	285	279	227	264	92
Test Article 0.05 µL/plate	279	284	314	292	102
Mitomycin-C	2,667	2,022	2,461	2,383	833 (+)
Water Control	267	268	312	282	N/A
Results With S-9 Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	348	395	395	379	N/A
Test Article 5 µL/plate	386	407	408	400	106
Test Article 1.6 µL/plate	380	413	389	394	104
Test Article 0.5 µL/plate	418	363	396	392	103
Test Article 0.16 µL/plate	386	381	382	383	101
Test Article 0.05 µL/plate	393	357	345	365	96
2-AA	3,067	2,914	3,105	3,029	798 (+)
Water Control	429	395	368	397	N/A

TA1535 (Number of Revertants): Percent of control results greater than (>) 300 qualify as positive according to the test acceptance criteria. The expected result for the chemical controls is included as ± in the parentheses ( ).

Results Without Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	36	13	20	23	N/A
Test Article 5 µL/plate	16	14	26	19	81
Test Article 1.6 µL/plate	26	27	18	24	103
Test Article 0.5 µL/plate	24	31	23	26	113
Test Article 0.16 µL/plate	22	26	21	23	100
Test Article 0.05 µL/plate	15	26	27	23	99
Sodium Azide	990	937	1,007	978	4,252 (+)
Water Control	29	22	26	26	N/A
Results With S-9 Activation					
Identification	Plate Count Results			Average	Percent of Control
Solvent Control	15	7	8	10	N/A
Test Article 5 µL/plate	9	17	18	15	147
Test Article 1.6 µL/plate	2	14	10	9	87
Test Article 0.5 µL/plate	13	9	11	11	110
Test Article 0.16 µL/plate	8	10	11	10	97
Test Article 0.05 µL/plate	7	12	11	10	100
2-AA	125	119	140	128	1,280 (+)
Water Control	20	26	21	22	N/A

Spot Test: Results for the spot tests recorded as + (positive) or - (negative). A positive result indicates that the material showed a zone of increased reversion at the inoculation site. A negative result indicates that the material did not show a zone of increased reversion or toxicity at the inoculation site.

Results Without Activation					
Identification	TA97A	TA98	TA100	TA102	TA1535
Test Article	-	-	-	-	-
Results With S-9 Activation					
Identification	TA97A	TA98	TA100	TA102	TA1535
Test Article	-	-	-	-	-

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

1) Tested strains for genotype verification and achieved the appropriate responses.
2) All chemical controls included in the test gave the appropriate responses.
3) The reversion rates for each tester strain were within the historical ranges as outlined in the protocol. Historical data are constantly changing, as new data from acceptable tests are created. Therefore, reversion rates may differ slightly.

Criteria for a Mutagen:

1) A reversion rate greater than 200% of the solvent control in strains TA97a, TA100 and TA102. A reversion rate greater than 300% of the solvent control in strains TA98 and TA1535.

2) Demonstration of a clear dose related response when dilutions are tested.

Criteria for a Non-Mutagen:

1) A reversion rate less than or equal to 200% of the solvent control in strains TA97a, TA100 and TA102. A reversion rate less than or equal to 300% of the solvent control in strains TA98 and TA1535.

2) No dose related response when dilutions are tested.

Procedure:

Broth Culture Preparation: Commercial culture discs were used to make frozen working cultures. The culture discs were added to nutrient broth and incubated at 37 ± 2°C on an orbital shaker until visibly turbid. The cultures were streaked for isolation onto MGPA master plates fortified with histidine, biotin, tetracycline, and ampicillin, as appropriate. One or more colonies from the master plates were used to inoculate nutrient broth and incubated at 37 ± 2°C on an orbital shaker for 5-7 hours. Aliquots of the cultures were frozen for later use. Frozen working cultures of each strain were used to inoculate nutrient broth for testing. The cultures were incubated for 4.5-5.5 hours on an orbital shaker at approximately 100 rpm. The titers of the cultures were determined and had concentrations of at least 10^8 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. The presence of the rfa mutation was verified by demonstrating sensitivity to crystal violet on nutrient agar plates. The histidine requirement was assured by plating onto minimal glucose agar plates with biotin and both with and without histidine.

Test Article Preparation: The test article was diluted in buffer supplied by the sponsor and tested at the following concentrations: 5 µL, 1.6 µL, 0.5 µL, 0.16 µL, 0.05 µL/plate. An aliquot of the solvent used was included and tested as the negative control. An aliquot of sterile USP water was also included and tested as an additional negative control. The concentrations tested were based on the OECD 471 highest recommended concentration for non-cytotoxic compounds.

Metabolic Activation System: The S-9 activation system was used to screen for the presence of mutagens from byproducts of the test article. 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 5-10 mL of 0.5 mM biotin and 0.5 mM histidine prior to use.

P.O. Box 571830 | Murray, UT 84157-1830 U.S.A. · 6280 South Redwood Road | Salt Lake City, UT 84123-6600 U.S.A.
www.nelsonlabs.com · Telephone 801 290 7500 · Fax 801 290 7998 · sales@nelsonlabs.com

Plate Incorporation Tests: Each test article concentration and the solvent control were tested both with and without S-9 metabolic activation. The S-9 specific chemical controls (2-aminofluorene and 2-aminoanthracene) were tested with S-9 metabolic activation only. Strain specific non-metabolic chemical controls were also included (Sodium Azide, Mitomycin-C and 4-nitro-0-phenylene-diamine). The non-metabolic chemical controls were tested without S-9 activation only.

Sterile 13 x100 mm test tubes were transferred to a waterbath held at 45 ± 2°C. Two mL aliquots of top agar were transferred to each test tube. Three replicates for each test article or control were prepared. The test organism and materials were added as specified in the table below:

Identification	Added/Replicate
Solvent Controls	2 mL top agar, 100 µL test organism and 100 µL of the solvent control.
Test Article	2 mL top agar, 100 µL test organism and 100 µL of each test article.
Chemical Controls	2 mL top agar, 100 µL test organism and 10 µL of the specified chemical.

Each replicate requiring S-9 metabolic activation had 0.5 mL of the prepared S-9 mix added.

The replicates were vortexed, poured onto MGPA plates, swirled to form an even layer and allowed to solidify. The plates were incubated for growth 37 ± 2°C for 48-72 hours.

Spot Tests: The test article was also analyzed using the spot method on plates with and without the S-9 activation system. Two mL aliquots of the top agar mixture and 100 µL of the appropriate test organism were added to minimal glucose agar plates. The plates were allowed to harden then 10 µL of the test article was added as a spot on the surface of the plate. The plates were incubated for growth of the organisms at 37 ± 2°C for 48-72 hours. Only the highest test article concentration was analyzed using the spot method.

Chemical Control Materials: The following chemical controls were used. The concentrations listed are the amount added per plate: 1.5 µg Sodium Azide, 2.5 µg Mitomycin-C, 20 µg 4-nitro-0-phenylene-diamine (NPD), 20 µg 2-aminofluorene (2-AF) and 7 µg 2-aminoanthracene (2-AA). The chemical controls were tested using the plate incorporation method only.

Historical Data 2013							
Strain TA97a without S9 Activation				Strain TA97a with S9 Activation			
Saline or Water	DMSO	PEG 400	NPD	Saline or Water	DMSO	PEG 400	2-AF
Minimum							
81	89	88	366	109	115	108	895
Maximum							
175	140	171	610	226	193	220	1520
Mean							
114	111	117	475	157	147	156	1120
Standard Deviation							
18	21	19	60	27	30	28	150

Historical Data 2013							
Strain TA98 without S9 Activation				Strain TA98 with S9 Activation			
Saline or Water	DMSO	PEG 400	NPD	Saline or Water	DMSO	PEG 400	2-AF
Minimum							
11	17	14	404	15	21	12	1826
Maximum							
56	28	53	1080	72	45	71	2885
Mean							
20	20	22	618	28	29	27	2339
Standard Deviation							
8	4	7	154	11	8	12	324

Historical Data 2013							
Strain TA100 without S9 Activation				Strain TA100 with S9 Activation			
Saline or Water	DMSO	PEG 400	Sodium Azide	Saline or Water	DMSO	PEG 400	2-AF
Minimum							
67	73	71	752	67	78	75	1228
Maximum							
157	129	165	1189	150	138	169	2159
Mean							
105	100	109	979	106	105	110	1721
Standard Deviation							
22	19	26	105	21	20	22	219

Historical Data 2013							
Strain TA102 without S9 Activation				Strain TA102 with S9 Activation			
Saline or Water	DMSO	PEG 400	Mitomycin C	Saline or Water	DMSO	PEG 400	2-AA
Minimum							
292	354	318	1377	360	412	365	1089
Maximum							
467	420	478	2298	545	517	539	4698
Mean							
383	381	393	1749	461	451	475	2143
Standard Deviation							
35	27	37	219	46	45	44	718

P.O. Box 571830 | Murray, UT 84157–1830 U.S.A. · 6280 South Redwood Road | Salt Lake City, UT 84123–6600 U.S.A.
www.nelsonlabs.com · Telephone 801 290 7500 · Fax 801 290 7998 · sales@nelsonlabs.com

Historical Data 2013							
Strain TA1535 without S9 Activation				Strain TA1535 with S9 Activation			
Saline or Water	DMSO	PEG 400	Sodium Azide	Saline or Water	DMSO	PEG 400	2-AA
Minimum							
11	9	10	702	8	10	6	48
Maximum							
61	29	63	1487	39	17	33	256
Mean							
24	19	25	1090	12	12	12	154
Standard Deviation							
11	7	12	214	5	3	5	72