CITREX, INC.

AMES II MUTATIONAL SPECTRA
Six Ames II Salmonella Strains, TA98, and TA1537

Test Article: Citrex B

Study Number: CIT-0900a
September, 2000

SPONSOR

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Scientist, Client Research and Operations

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Date: September 16, 2000

CLIENT RESEARCH LABORATORY REPORT

Citrex, Inc.

AMES II ASSAY Mutational Spectra

(TA7001, TA7002, TA7003, TA7004, TA7006, TA98 and TA1537 Salmonella Strains)

Test Article: Citrex B

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1. SUMMARY

Test article Citrex B was tested in the Ames II Mutational Spectra bacterial mutagenesis assay, using a *Salmonella typhimirium* host and 6 unique genetic reversion targets in the bacterial genome. The assay also includes the traditional Ames tester strains TA98 and TA1537. The strains in the Mutational Spectra assay are all histidine auxotrophs, and mutagenesis at specific bases in each strain will lead to reversion of the strain to histidine prototrophy. Details of each strain (genotype) are given in section 3.2.

Assays for each *Salmonella* strain were performed in triplicate. A 100 µg/ml stock of the test article was prepared in sterile water. Doses of test article Citrex B in the Ames II Mutational Spectra Assay were: 0, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 µg/ml, with concurrent solvent (water) negative controls which gave revertant numbers and frequencies within expected ranges for each strain (for expected ranges see Table 1). Testing was performed in the absence of S9 fraction.

Under the conditions listed above, and using criteria employed to evaluate Ames II Mutational Spectra Assay data, test article Citrex B demonstrated no mutagenic activity in the strains TA7001, TA7002, TA7003, TA7004, TA7005, TA7006, TA98, and TA1537.

2. INTRODUCTION

The purpose of this study was to assess the mutagenic potential of test article Citrex B in the Ames II Mutational Spectra Assay. Each of the six TA7000 series strains, and TA98 and TA1537 *Salmonella* strains were employed in the assay, permitting the measurement of 6 different base-substitutions (transitions and transversions), and a frameshift mutation. The assay was performed in the absence of exogenous biotransformation enzymes (S9 fraction). The assay is a microplate liquid culture modification of that reported in Gee *et al.* (1994), and is described in further detail in Section 3.6 and Appendix I.

3. MATERIALS & METHODS

3.1 Test Article

Test article Citrex B arrived in a small white plastic container with a white screw-top lid. The container's label contained the following information:

Special Nutrients, Inc.
1394 Coral Way Miami, FL 33145
Tel: (305) 857-9839 Fax (305) 857-6973
Citrex B
España
Date: 04/08/2000

The concentration of the test article was communicated verbally on July 28, 2000 as 1.15 kg/L.

The test article was a yellow viscous liquid, and was stored in the dark at room temperature.

3.2 Bacterial Strains

Table 1 lists the Ames II strains.

Table 1. AMES II Strains

Strain	Target	Mutation [a]	rfa[b]	uvrB[c]	pKM101[d]	QC rev/48 wells[e]
TA7001	bp subst.[f]	A:T→G:C	✓	✓	✓	0 - 3
TA7002	bp subst.	T:A→A:T	✓	✓	✓	0 - 5
TA7003	bp subst.	T:A→G:C	✓	✓	✓	0 - 1
TA7004	bp subst.	G:C→A:T	✓	✓	✓	0 - 10
TA7005	bp subst.	G:C→T:A	✓	✓	✓	0 - 10
TA7006	bp subst.	C:G→G:C	✓	✓	✓	0 - 5
Mix	bp.subst.	All above	✓	✓	✓	0 - 10
TA98	frameshift	+G:C	✓	✓	✓	0 - 17
TA1537	frameshift	+G:C	✓	✓	×	0 - 17

[a] Base change detected by this strain.
[b] Defective lipopolysaccharide cell wall, permitting greater access for larger molecules.
[c] Defective in DNA repair.
[d] Plasmid which enhances error-prone repair & confers Ampicillin resistance.
[e] Spontaneous revertants required for Quality Control acceptance. Slightly higher spontaneous levels are, in many cases still passed on variance.
[f] Base pair substitution detected.
✓ present, × absent.

3.3 Metabolic Activation

The assay described in this report was peformed in the absence of S9 fraction and therefore did not include exogenous metabolic activation.

3.4. Positive Controls

The following positive control chemicals were used in assessing the performance of the reported Ames II Assays.

Table 2. Positive Controls

Ames II Strain	Control Chemical	Conc.
TA7001	N-4-Aminocytidine (N4A)	12.5 – 100 µg/ml
TA7002	Methanesulfonic Acid Methyl Ester (MMS)	125 – 1000 µg/ml
TA7003	Streptonigrin	1.25 – 10 ng/ml
TA7004	4-Nitroquinoline-N-Oxide (4NQO)	62.5 – 500 ng/ml
TA7005	4-Nitroquinoline-N-Oxide	62.5 – 500 ng/ml
TA7006	4-Nitroquinoline-N-Oxide	62.5 – 500 ng/ml
TA98	2-Nitrofluorene (2NF)	0.25-2.0 µg/ml
TA1537	2-aminoanthracene (2AA)	0.6-5.0 µg/ml

3.5. Test Article Preparation

A 25X concentrated stock solution of the test article was prepared in sterile water as listed below. The test article was prepared immediately prior to use.

Table 3. Test Article Preparation

Test Article	Solvent	Stock Concentration
Citrex B	Water	100 µg/ml

3.6. Ames II Assay

The assay protocol is described in Appendix I.

Test article Citrex B was tested in the TA7000 series, TA98 and TA1537 *Salmonella* strains at 9 dose levels (including a zero dose). Each dose was performed in triplicate using independent cultures.

The doses employed were 0, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 µg/ml.

3.6.1. *Dosing Protocol (no S9)*

Into one well of a 24 well plate (one well/strain/dose/replicate) 0.215 ml of Exposure Medium was aliquoted. To this was added 0.025 ml of culture, giving a combined volume of 0.240 ml. To each of these cultures, 0.010 ml of appropriately diluted test article was added for a total volume of 0.250 ml. This mixture was then incubated for 90 minutes at 37°C with agitation at 250 rpm.

At the conclusion of the 90 minute incubation, each well received 2.8 ml of *Salmonella* Indicator Medium and was mixed briefly before being distributed in 0.05 ml aliquots to 48 wells of a 384-well microtiter plate. One plate was used/strain/replicate.

Plates were then incubated at 37°C for 48 hours, after which yellow (positive) wells were counted.

3.6.2. *Determination of Positive Wells*

From triplicate data sets, the mean number of positive wells for each strain at each dose was calculated.

3.6.3. *Determination of Fold Increase*

The fold increase of bacterial revertant colonies compared to the background (zero dose) revertant colony number was determined by dividing the mean number of colonies at each dose by that at the zero dose. Student's *t*-tests were used to determine significance (at the $\alpha = 0.05$ level) for fold inductions greater than 3.0. The Student's t-test was performed to test the null hypothesis for every concentration of compound in comparison to the zero-dose control. Because the samples have different variance, the following formula was used:

$$t = \frac{\left[\bar{x}_1 - \bar{x}_2\right]}{\sqrt{\left[\frac{S_1^2}{n_1}\right] + \left[\frac{S_2^2}{n_2}\right]}}$$

where:

$\bar{x}_1$ = the mean colony count for sample 1 (zero dose control)
$\bar{x}_2$ = the mean colony count for sample 2 (concentration X)
S_1^2 = the variance of colony count 1
S_2^2 = the variance of colony count 2
n_1 = the number of replicate colony counts for count 1 ($n = 3$)
n_2 = the number of replicate colony counts for count 2 ($n = 3$)

Since the t-test was used to evaluate means *greater* than the control value, reference was made to a table of critical values for the Student's t-test, which records an area of α in the right hand tail only.

The degrees of freedom value was determined by the smaller of the values n_1-1 or n_2-1, both of which equal 2.
Although statistical analyses have been applied to all data collected, fold increases in revertant numbers in the Ames II Assay are not classified as positive if less than 3.0. Below this fold increase value, the data are unreliable with respect to determining mutagenicity. To be classified as a mutagen, a compound is therefore required to yield a reproducible fold increase of greater than 3.0, or to show a clear dose response.

3.6.4. *Data Analysis*

The mean number of positive wells and fold increase was determined as described above. A positive result in the Ames II Assay requires a fold increase value of greater than 3.0.

3.6.5. *Archived Data*

Raw data for this study are located in Xenometrix laboratory notebook(s) as follows:

Table 4. Archived Data

Notebook	Page(s)
179/CRL	85 – 90, 92, 94 - 96
180/CRL	5, 10 - 13

4. RESULTS

4.1 Test Article Result Summaries

Table 5. Test Article Citrex B: TA7001

TA7001

Conc. (µg/ml)	n[a]	mean # pos. wells[b]	SD[c]	Fold Increase[d]	t-test (2DF) alpha = 0.05[e]
0	8	0.25	1.31		
0.03	3	0.00	0.00	0.00	0.54
0.06	3	0.00	0.00	0.00	0.54
0.125	3	1.33	0.58	5.33	1.90
0.25	3	0.00	0.00	0.00	0.54
0.5	3	0.00	0.00	0.00	0.54
1.0	3	0.67	1.15	2.67	0.51
2.0	3	0.00	0.00	0.00	0.54
4.0	3	0.00	0.00	0.00	0.54

[a] number of replicates
[b] mean number of positive wells counted over three replicate 48-well portions of a 384-well plate
[c] standard deviation
[d] mean number of positive wells at each dose, divided by the mean number of positive wells at the zero dose control.
[e] Student's t-test significant at the 0.05 level if calculated value is greater than 2.92 (2 degrees of freedom)

Although inductions of 5.33- and 2.67-fold were observed at 0.125 and 1.0 µg/ml, these inductions were not statistically significant and a clear dose-response was not observed. Thus, test article Citrex B was not mutagenic at the doses tested when assayed with the Ames II TA7001 strain in the absence of S9 fraction. Plate counts for Table 5 are shown in Appendix II.

Table 6. Test Article Citrex B: TA7002

TA7002

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	0.25	0.46		
0.03	3	0.67	1.15	2.67	0.61
0.06	3	0.00	0.00	0.00	1.53
0.125	3	0.33	0.58	1.33	0.22
0.25	3	0.00	0.00	0.00	1.53
0.5	3	0.33	0.58	1.33	0.22
1.0	3	0.67	0.58	2.67	1.12
2.0	3	0.00	0.00	0.00	1.53
4.0	3	0.00	0.00	0.00	1.53

Test article Citrex B showed no evidence of mutagenicity when assayed with TA7002 in the absence of S9. Plate counts for Table 6 are shown in Appendix II.

Table 7. Test Article Citrex B: TA7003

TA7003

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	0.00	0.00		
0.03	3	0.33	0.58	-	1.00
0.06	3	0.00	0.00	-	-
0.125	3	0.00	0.00	-	-
0.25	3	0.00	0.00	-	-
0.5	3	0.33	0.58	-	1.00
1.0	3	1.00	1.73	-	1.00
2.0	3	0.00	0.00	-	-
4.0	3	0.00	0.00	-	-

Test article Citrex B showed no evidence of mutagenicity when assayed with TA7003 in the absence of S9. Plate counts for Table 7 are shown in Appendix II.

Table 8. Test Article Citrex B: TA7004

TA7004

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	3.88	1.31		
0.03	3	4.33	1.53	1.12	0.46
0.06	3	5.67	0.58	1.46	3.14
0.125	3	4.33	3.06	1.12	0.25
0.25	3	4.67	1.53	1.20	0.79
0.5	3	6.00	1.00	1.55	2.87
1.0	3	2.67	2.31	0.69	0.86
2.0	3	2.33	1.53	0.60	1.55
4.0	3	3.33	0.58	0.86	0.95

A 1.46-fold increase in mean number of positive wells observed at 0.06 µg/ml was statistically significant; however, this induction was lower than the 3-fold induction threshold for reliability and no dose-response function was observed. Therefore, test article Citrex B was not mutagenic when assayed with TA7004 in the absence of S9. Plate counts for Table 9 are shown in Appendix 2.

Table 9. Test Article Citrex B: TA7005

TA7005

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	5.38	3.29		
0.03	3	5.00	1.00	0.93	0.29
0.06	3	7.67	3.21	1.43	1.05
0.125	3	6.67	0.58	1.24	1.07
0.25	3	7.33	1.53	1.36	1.34
0.5	3	4.67	0.58	0.87	0.59
1.0	3	3.67	1.15	0.68	1.27
2.0	3	5.67	3.79	1.05	0.12
4.0	3	4.67	2.08	0.87	0.42

Test article Citrex B showed no evidence of mutagenicity when assayed with TA7005 in the absence of S9. Plate counts for Table 9 are shown in Appendix II.

Table 10. Test Article Citrex B: TA7006

TA7006

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	1.75	1.79		
0.03	3	1.33	1.53	0.76	0.38
0.06	3	1.00	1.00	0.57	0.88
0.125	3	1.00	0.00	0.57	1.19
0.25	3	1.33	1.15	0.76	0.45
0.5	3	2.33	1.53	1.33	0.54
1.0	3	1.33	0.58	0.76	0.58
2.0	3	0.67	0.58	0.38	1.52
4.0	3	1.00	1.00	0.57	0.88

Test article Citrex B was not mutagenic when assayed with TA7006 in the absence of S9 fraction. Plate counts for Table 10 are shown in Appendix II.

Table 11. Test Article Citrex B: TA98

TA98

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	7.50	1.31		
0.03	3	8.00	3.00	1.07	0.28
0.06	3	7.33	0.58	0.98	0.29
0.125	3	7.00	2.00	0.93	0.40
0.25	3	6.67	0.58	0.89	1.46
0.5	3	7.33	2.31	0.98	0.12
1.0	3	8.67	2.52	1.16	0.77
2.0	3	5.33	3.06	0.71	1.19
4.0	3	6.67	1.53	0.89	0.84

Test article Citrex B showed no evidence of mutagenicity when assayed with TA98 in the absence of S9. Plate counts for Table 11 are shown in Appendix II.

Table 12. Test Article Citrex B: TA1537

TA1537

Conc. (µg/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	8	20.63	4.37		
0.03	3	18.33	2.08	0.89	1.17
0.06	3	21.67	7.09	1.05	0.24
0.125	3	21.67	6.03	1.05	0.27
0.25	3	28.00	3.61	1.36	2.84
0.5	3	22.00	5.57	1.07	0.39
1.0	3	13.00	1.73	0.63	4.14
2.0	3	24.00	3.46	1.16	1.34
4.0	3	21.00	5.00	1.02	0.11

Test article Citrex B was not mutagenic when assayed with TA1537 in the absence of S9 fraction. Plate counts for Table 10 are shown in Appendix II.

4.2 Positive Control Results

Positive control results, shown as the number of revertant wells, are presented in the following tables.

Table 13a. Positive Controls (TA7001 – TA7003)

Positive Controls

TA7001		TA7002		TA7003	
N4A (µg/ml)	# revertants	MMS (ug/ml)	# revertants	Streptonigrin (ng/ml)	# revertants
12.5	1	125	0	1.25	0
25	2	250	1	2.5	1
50	8	500	4	5	1
100	19	1000	6	10	2

Table 13b. Positive controls (TA7004 – TA7006)

Positive Controls

TA7004		TA7005		TA7006	
4NQO (ng/ml)	# revertants	4NQO (ng/ml)	# revertants	4NQO (ng/ml)	# revertants
62.5	5	62.5	17	62.5	7
125	13	125	42	125	11
250	24	250	48	250	24
500	34	500	48	500	30

Table 13c. Positive Controls (TA98 and TA1537)

Positive Controls

TA98		TA1537	
2NF (µg/ml)	# revertants	2NF (µg/ml)	# revertants
0.25	3	0.25	14
0.5	10	0.5	23
1	25	1	23
2	22	2	45

5. DISCUSSION

All negative controls (zero dose solvent controls) run concurrently with the assays gave revertant colony numbers within expected limits. TA1537 gave slightly higher revertant colony numbers but passed QC on variance.

Quality Control appropriate positive controls gave revertant colony numbers within expected limits.

Exposure of the Ames II strains to Citrex B did not produce significant increases in number of revertant wells compared to negative controls, except in a few isolated cases. In situations in which significant fold-inductions occurred, the fold-induction was below the critical threshold level of 3.0 and was seen in the absence of a dose-response and therefore did not meet the criteria for a conclusion of mutagenicity.

6. CONCLUSION

Using the experimental conditions outlined in this report, and at the concentrations tested, test article Citrex B is not mutagenic in the Ames II strains: TA7001, TA7002, TA7003, TA7004, TA7005, TA7006, or in TA98 or TA1537 in the absence of metabolic activation by S9 fraction.

7. REFERENCES

Gee, P., Maron, D., and Ames, B.N., (1994) Detection and classification of mutagens: A set of base-specific *Salmonella* tester strains *Proc. Natl. Acad. Sci. (USA)* **91**, 11606-610.

Vennit, S., Crofton-Sleigh, C., and Foster, R., (1984) In: *Mutagenicity Testing*, pp45 (S. Vennit and J.M. Parry, eds), IRL Press, Oxford, UK.

Appendix I: Ames II Assay Outline

General Description

The Ames II Assay consists of six new *Salmonella typhimurium* tester strains, each of which detects only one of the six possible base pair substitution mutation types, allowing the generation of mutational spectra data without the need for DNA sequence analysis. Because these strains revert only by specific mutational events, spontaneous reversion frequencies are approximately 10 to 100-fold lower than those typically observed with traditional *Salmonella* tester strains such as TA100 or TA102, which have a range of possible reversion pathways. Ames II strain reversion frequencies are shown in Table 14. The minimal genetic complementation between the six TA7000 series strains means that they may be combined in a single assay in order to screen for moderate to strong mutagens if there is no requirement for mutational spectra data. The tester strains TA98 and TA1537 are included in the full Ames II Assay (Ames II complete) for the detection of frameshift-inducing mutagens. However, in the rapid Ames II screen (Ames II (Mix + TA98)), only the more sensitive TA98 is used.

The new *Salmonella* tester strains TA7001-TA7006 have been modified for maximum sensitivity to test compound. These modifications include:

- Deletion of the excision repair gene *uvrB*. This mutation prevents the removal of bulky adducts from DNA.
- Mutation of a gene required for synthesis of the bacterial cell wall (*rfa*) which increases permeability of *Salmonella* to test compound.
- Introduction of the episome pKM101, which carries the *umuDC* homologues *mucA/B*. These gene products increase the cell's ability to perform mutagenic lesion bypass repair during DNA replication.

Table 14. Ames II Strains

Strain	Target	Mutation [a]	rfa	uvrB	pKM101	rev/10^8 [b]
TA7001	bp subst. [c]	A:T→G:C	✓	✓	✓	0 - 5
TA7002	bp subst.	T:A→A:T	✓	✓	✓	0 - 9
TA7003	bp subst.	T:A→G:C	✓	✓	✓	0 - 1
TA7004	bp subst.	G:C→A:T	✓	✓	✓	0 - 20
TA7005	bp subst.	G:C→T:A	✓	✓	✓	0 - 20
TA7006	bp subst.	C:G→G:C	✓	✓	✓	0 - 5
Mix	bp.subst.	All above	✓	✓	✓	0 - 20
TA98	frameshift	+GC	✓	✓	✓	0 - 35
TA1537	frameshift	+GC	✓	✓	×	0 - 35

[a] Base change detected by this strain
[b] Revertants per 10^8 viable cells
[c] Base-pair substitutions detected
✓ The given characteristic is present in this strain
× The given characteristic is absent from this strain

Assay Procedure

Because of the low spontaneous reversion frequencies for the TA7000 strains and their associated mixture, the assay has been converted to a modified fluctuation test using 384 well (48 wells per sample, per dose) microtiter plates. The frameshift tester strains are not combined with the Ames II Mix, but are assayed concurrently in microtiter plates.

The modified Ames II fluctuation test, in brief, is as follows:

1. Pre-growth of tester strains overnight in oxoid broth.
2. A 90 minute incubation (10^8 cells x 1 generation) in Exposure Medium with limiting histidine (1.0 mg/ml) in the presence of toxicant and S9 if employed.
3. Dilution and plating of cells in medium which selects for revertants. This medium contains a pH indicator dye that turns color from purple to yellow upon colony growth.
4. Incubation of the microtiter plates for 48 hours to allow growth of revertant colonies.
5. Scoring of microtiter plates for positive (yellow) wells, data entry, and evaluation of mutagenic potential.

Appendix II: Raw Data

Plate counts

Table 15. Test Article Citrex B: TA7001

TA7001

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	0	0	0
0.06	0	0	0
0.125	1	1	2
0.25	0	0	0
0.5	0	0	0
1.0	0	0	2
2.0	0	0	0
4.0	0	0	0

Table 16. Test Article Citrex B: TA7002

TA7002

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	0	0	2
0.06	0	0	0
0.125	1	0	0
0.25	0	0	0
0.5	0	0	1
1.0	0	1	1
2.0	0	0	0
4.0	0	0	0

Table 17. Test Article Citrex B: TA7003

TA7003

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	0	1	0
0.06	0	0	0
0.125	0	0	0
0.25	0	0	0
0.5	1	0	0
1.0	3	0	0
2.0	0	0	0
4.0	0	0	0

Table 18. Test Article Citrex B: TA7004

TA7004

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	4	6	3
0.06	5	6	6
0.125	5	1	7
0.25	3	6	5
0.5	5	6	7
1.0	4	4	0
2.0	1	2	4
4.0	4	3	3

Table 19. Test Article Citrex B: TA7005

TA7005

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	4	5	6
0.06	9	10	4
0.125	7	7	6
0.25	9	7	6
0.5	5	5	4
1.0	5	3	3
2.0	3	10	4
4.0	7	4	3

Table 20. Test Article Citrex B: TA7006

TA7006

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	0	1	3
0.06	2	1	0
0.125	1	1	1
0.25	2	2	0
0.5	4	2	1
1.0	1	1	2
2.0	1	0	1
4.0	1	2	0

Table 21. Test Article Citrex B: TA98

TA98

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	5	11	8
0.06	7	7	8
0.125	7	5	9
0.25	7	7	6
0.5	6	10	6
1.0	6	11	9
2.0	6	2	8
4.0	8	7	5

Table 22. Test Article Citrex B: TA1537

TA1537

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
0.03	16	19	20
0.06	23	28	14
0.125	16	21	28
0.25	25	32	27
0.5	23	16	27
1.0	14	14	11
2.0	22	22	28
4.0	16	21	26

Table 23. Spontaneous Revertants (Negative Controls); n = 8

Spontaneous Revertants (Negative Controls)

TA7001	TA7002	TA7003	TA7004	TA7005	TA7006	TA98	TA1537*
0	1	0	4	1	3	6	23
1	0	0	3	7	4	10	27
0	0	0	4	11	1	6	12
0	0	0	2	8	2	8	21
0	1	0	8	4	0	7	23
1	0	0	5	2	4	8	21
0	0	0	3	4	0	8	20
0	0	0	2	6	0	7	18

*Spontaneous revertant counts were slightly higher than those generally observed for this strain, but passed QC on variance.