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Huntingdon Life Sciences

[redacted]

BACTERIAL MUTATION ASSAY

Company Sanitized. Does not contain TSCA CBI

Report

BACTERIAL MUTATION ASSAY

Sponsor

DuPont Specialty Chemicals,
Jackson Laboratory,
Chambers Works,
Deepwater,
NJ 08023,
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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, with the exception stated below, and I consider the data generated to be valid.

The United Kingdom Good Laboratory Practice Regulations 1997 (Statutory Instrument No 654).

EC Council Directive 87/18/EEC of 18 December 1986 (Official Journal No L 15/29).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

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Kenneth May, B.Sc., C.Biol., M.I.Biol.,
Study Director,
Huntingdon Life Sciences Ltd.

1 October 1998

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study:

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol	3 August 1998	3 August 1998
Process Based Inspections		
Audit	8 January 1998	8 January 1998
Plate Scoring	6 April 1998	6 April 1998
Treatment	15 April 1998	15 April 1998
Formulation	14 July 1998	14 July 1998
Report	2 September 1998	2 September 1998

Protocol: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections and audits of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

G.Goddard

28TH SEPTEMBER 1998

G Goddard
Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

Date

SUMMARY

In this *in vitro* assessment of the mutagenic potential of Zonyl® FS-62, histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan dependent mutant of *Escherichia coli*, strain CM891 (WP2uvrA/pKM101), were exposed to the test substance diluted in purified water, which was also used as a negative control.

Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix). The first was a standard plate incorporation assay, the second involved a pre-incubation stage.

Concentrations of up to 5000 µg/plate were tested in the mutation tests. This is the standard limit concentration recommended in the regulatory guidelines this assay follows. Other concentrations used were a series of *ca* half-$\log_{10}$ dilutions of the highest concentration. No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of [redacted] in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that, when tested in purified water, [redacted] shows no evidence of mutagenic activity in this bacterial system.

INTRODUCTION

This report describes a study designed to assess the mutagenic potential of [redacted] in a bacterial system. The study was conducted in compliance with the following guidelines:

OECD Guidelines for Testing of Chemicals. (1997) No. 471: Genetic Toxicology: Bacterial Reverse Mutation Test.

EEC Annex to Directive 92/69/EEC. (1992) Part B : Methods for Determination of Toxicity, B.13. Other effects - Mutagenicity: *Escherichia coli* - Reverse Mutation Assay. *O.J.* No. L 383 A, 157.

EEC Annex to Directive 92/69/EEC. (1992) Part B : Methods for Determination of Toxicity, B.14. Other effects - Mutagenicity: *Salmonella typhimurium* - Reverse Mutation Assay. *O.J.* No. L 383 A, 160.

US EPA 40 CFR Part 799 (1997) Toxic Substances Control Act Test Guidelines - Sub-section 799.9510, TSCA bacterial reverse mutation test. Federal Register, Vol. 62, No. 158.

The method described was also designed to comply with ICH (1996 & 1997), and followed the recommendations of the United Kingdom Environmental Mutagen Society (Gatehouse *et al* 1990).

The *in vitro* technique described by Ames and his co-workers, (Ames, McCann and Yamasaki 1975, Maron and Ames 1983) enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance. Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine deficient medium (supplemented with biotin, since these strains are also incapable of biotin synthesis).

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* which are incapable of synthesising the amino acid tryptophan required for growth.

The strains used carry additional mutations which render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition three of them possess a plasmid (pKM101) which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore the bacteria and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzymic activity.

The protocol was approved by Huntingdon Life Sciences Management on 7 July 1998, the Sponsor on 17 July 1998 and by the Study Director on 31 July 1998.

The experimental phase of the study was conducted between 3 and 17 August 1998.

TEST SUBSTANCE

Identity:	[redacted]
Chemical name:	[redacted]
Appearance:	[redacted]
Storage conditions:	Room temperature
Lot number:	[redacted]
Expiry date:	2 Years from date of receipt
Purity:	[redacted]
Date received:	23 June 1998

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The following strains were used:-

S. typhimurium TA1535: contains a histidine missense mutation (*his*G46) but is also deficient in a DNA repair system (*uvr*B) and has a defective lipopolysaccharide coat on the cell wall (*rfa* mutation). It is reverted by many agents causing base-pair substitutions, but is not sensitive to frameshift mutagens.

S. typhimurium TA100: is the same as TA1535 but contains a resistance transfer factor conferring ampicillin resistance and increasing sensitivity to some mutagens (plasmid pKM101). In addition to base-pair substitutions, it is also able to detect certain frameshift mutagens.

S. typhimurium TA1537: bears a histidine frameshift mutation (*his*C3076). Like TA1535, it is defective in a DNA repair system and lipopolysaccharide coat. It is sensitive to agents causing frameshift mutations involving insertion or deletion of a single base-pair.

S. typhimurium TA98: contains another histidine frameshift mutation (*his*D3052). Again it has a defective DNA repair system and lipopolysaccharide coat but also contains the pKM101 plasmid. It is reverted by agents causing deletion of two adjacent base-pairs (double frameshift mutations), but not by simple alkylating agents causing base-pair substitutions.

E. coli CM891: (WP2uvrA/pKM101) contains an ochre mutation. It is reverted by many agents causing A-T base-pair substitutions at the *trp*E locus or by G-C base-pair substitutions in transfer RNA loci elsewhere in the chromosome. It is also deficient in a DNA repair system (*uvr*A), and is more readily reverted by certain mutagens than its parent strain WP2. It also contains the pKM101 plasmid.

The strains of *S. typhimurium* were obtained from Professor B.N. Ames, University of California, Berkeley, California, USA.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were aliquots of nutrient broth cultures and were stored at -80°C. Dimethyl sulphoxide (DMSO) was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light and the pKM101 plasmid which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens was also assessed.

For use in tests an aliquot of frozen culture was added to 25 ml of nutrient broth (Merck No. 2) and incubated, with shaking, at 37°C for 10 hours. These cultures provided at least 10^9 cells per ml which were measured by spreading aliquots (0.1 ml) of a 10^{-6} dilution of the overnight cultures on the surface of plates of nutrient agar.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	*N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)
Supplier:	Sigma Chemical
Lot number:	20F-0235
Purity:	≥97%
Appearance:	Pale yellow crystalline powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade, ≥99.9% pure)
Concentration:	5 µg/plate for strain TA1535 3 µg/plate for strain TA100 2 µg/plate for strain CM891

Identity:	9-Aminoacridine
Supplier:	Sigma Chemical
Batch number:	108C-0358
Purity:	99%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade, ≥99.9% pure)
Concentration:	30 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
Supplier:	Aldrich Chemical Company
Batch number:	012867
Purity:	98%
Appearance:	Beige powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade, ≥99.9% pure)
Concentration:	1 µg/plate for strain TA98

In the presence of S9 mix

Identity:	2-Aminoanthracene
Supplier:	Aldrich Chemical Company
Batch number:	52234-024
Purity:	96%
Appearance:	Green powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade, ≥99.9% pure)
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain CM891

Identity:	Benzo[a]pyrene
Supplier:	Aldrich Chemical Company
Batch number:	07778-105
Purity:	98%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade, ≥99.9% pure)
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Harlan Olac Ltd
Age:	7-8 weeks
Weight:	<300 g

S9 fraction was prepared from a group of *ca* 10 animals. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in Arachis oil at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats were killed and their livers aseptically removed.

The following steps were carried out at 0-4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to an Ultra-Turrax homogeniser. Following preparation, the homogenate was centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C until required. The efficacy of each batch of S9 fraction was tested in a bacterial mutation assay with the mutagenic precursors 7,12-dimethylbenzanthracene and 2-aminoanthracene before use. The sterility was also checked.

Date of preparation: 9 June 1998

PREPARATION OF S9 MIX

S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

SELECTION OF SOLVENT AND FORMULATION OF TEST SUBSTANCE

The solubility of the test substance was assessed at 50 mg/ml in purified water, in which it dissolved following warming to 35-40°C with gentle stirring. Therefore purified water (obtained by the reverse osmosis of tap water) was used as the solvent for this study.

All concentrations cited in this report are expressed in terms of pure [redacted] i.e. correction was made for the purity of [redacted]

MUTATION TEST PROCEDURE

First test

The test substance was added to cultures of the five tester strains at seven concentrations separated by *ca* half-$\log_{10}$ intervals. The highest concentration of [redacted]ested was 50 mg/ml in the chosen solvent, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration recommended in the regulatory guidelines this assay follows. The negative control was the chosen solvent, purified water. The appropriate positive controls were also included.

An aliquot of 0.1 ml of a 10 hour bacterial culture and 0.5 ml S9 mix or 0.5 ml 0.1 M phosphate buffer (pH 7.4) were placed in glass tubes. An aliquot of 0.1 ml of the test solution was added, followed immediately by 2 ml of molten agar containing 0.5mM histidine/biotin/tryptophan. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing 25 ml minimal agar. Each petri dish was individually labelled with a unique code corresponding to a sheet, identifying the dish's contents. Three petri dishes were used for each concentration. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and phosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the second test would be the same as that used in the first. If toxic effects were observed a lower concentration may be chosen. It should be ensured that if a lower concentration was chosen, signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained.

Second test

As a clear negative response was obtained in the first test, a variation to the test procedure was used for the second. The variation used was the pre-incubation assay in which the tubes were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay. 5000 µg/plate was again chosen as the top concentration, but only five concentrations were used.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and the stability of the test substance in the solvent were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

For a test to be considered valid the mean of the solvent control revertant colony numbers for each strain should lie in the range stated in the appropriate Standard Operating Procedure. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

The mean number of revertant colonies for all treatment groups were compared with those obtained for the solvent control groups. The mutagenic activity of a test substance was assessed by applying the following criteria:

a) If treatment with a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent controls, with some evidence of a positive dose-relationship, in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.

b) If treatment with a test substance does not produce reproducible increases of at least 1.5 times the concurrent solvent controls in either mutation test it is considered to show no evidence of mutagenic activity in this test system. No statistical analysis is performed.

c) If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response given in paragraphs a) and b), additional testing may be performed in order to resolve the issue of the test substance's mutagenic activity in this test system. Should an increase in revertant colony numbers then be observed which satisfies paragraph (a) the substance is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.

If no clear "positive" response can be obtained, the test data may be subjected to analysis to determine the statistical significance of any observed increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be analysis of variance followed by Dunnett's test.

MAINTENANCE OF RECORDS

All experimental data arising from the study (including documentary raw data, records and other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Huntingdon Life Sciences shall retain the materials in its archive for a period of five years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials, subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences shall also retain a copy of the final report in its archive indefinitely.

RESULTS

The results obtained with [redacted] and positive control compounds are presented in Tables 1 to 5. The mean values quoted have been corrected to the nearest whole number.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination.

The total colony counts on nutrient agar plates (see Tables) confirmed the viability and high cell density of the cultures of the individual organisms.

The mean revertant colony counts for the solvent controls were within the ranges stated in the appropriate Standard Operating Procedure or quoted by Gatehouse *et al* (1990). Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

FIRST TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to [redacted] at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to [redacted]. A top exposure concentration of 5000 µg/plate was therefore selected for use in the second test.

SECOND TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to [redacted] at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to [redacted]

CONCLUSION

It is concluded that, when tested in purified water, [redacted] shows no evidence of mutagenic activity in this bacterial system.

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MARON, D.M. and AMES, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.* **113**, 173.

TABLE 1

Results obtained with *Salmonella typhimurium* TA98 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 1				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted](5000); sterility check	-	0	0	0	0	0
3	[redacted](5000)	+	45	37	44	42	4
4	[redacted](1500)	+	44	35	43	41	5
5	[redacted](500)	+	31	37	42	37	6
6	[redacted](150)	+	31	34	36	34	3
7	[redacted](50)	+	38	38	31	36	4
8	[redacted](15)	+	32	27	34	31	4
9	[redacted]5)	+	41	32	48	40	8
10	Purified water (0.1 ml)	+	38	43	43	41	3
11	[redacted](5000)	-	41	34	42	39	4
12	[redacted](1500)	-	24	41	39	35	9
13	[redacted](500)	-	34	34	45	38	6
14	[redacted](150)	-	37	38	35	37	2
15	[redacted]50)	-	44	36	35	38	5
16	[redacted]15)	-	41	50	34	42	8
17	[redacted]5)	-	45	30	31	35	8
18	Purified water (0.1 ml)	-	43	39	34	39	5
19	Benzo[a]pyrene (5)	+	554	580	562	565	13
20	2-Nitrofluorene (1)	-	184	284	218	229	51
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	119	143	129	130	12
sd	Standard deviation						

TABLE 1 - continued

Results obtained with *Salmonella typhimurium* TA98 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means Test 2 (with pre-incubation) A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	Zonyl® FS-62 (5000); sterility check	-	0	0	0	0	0
3	[redacted] (5000)	+	34	38	37	36	2
4	[redacted] (1500)	+	37	42	34	38	4
5	[redacted] (500)	+	37	42	27	35	8
6	[redacted] (150)	+	39	42	42	41	2
7	[redacted] (50)	+	37	20	39	32	10
8	Purified water (0.1 ml)	+	36	37	36	36	1
9	[redacted] (5000)	-	35	38	29	34	5
10	[redacted] (1500)	-	28	41	38	36	7
11	[redacted] (500)	-	32	37	37	35	3
12	[redacted] (150)	-	30	44	37	37	7
13	[redacted] (50)	-	35	37	39	37	2
14	Purified water (0.1 ml)	-	43	32	41	39	6
15	Benzo[a]pyrene (5)	+	478	471	484	478	7
16	2-Nitrofluorene (1)	-	246	306	304	285	34
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	116	112	110	113	3
sd	Standard deviation						

TABLE 2

Results obtained with *Salmonella typhimurium* TA100 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 1				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted] (5000); sterility check	-	0	0	0	0	0
3	[redacted] (5000)	+	109	93	89	97	11
4	[redacted] (1500)	+	100	90	94	95	5
5	[redacted] (500)	+	106	96	85	96	11
6	[redacted] (150)	+	100	109	102	104	5
7	[redacted] (50)	+	101	100	103	101	2
8	[redacted] (15)	+	86	92	103	94	9
9	[redacted] (5)	+	87	102	101	97	8
10	Purified water (0.1 ml)	+	110	100	96	102	7
11	[redacted] (5000)	-	94	114	88	99	14
12	[redacted] (1500)	-	99	100	86	95	8
13	[redacted] (500)	-	106	90	104	100	9
14	[redacted] (150)	-	92	110	92	98	10
15	[redacted] (50)	-	106	108	106	107	1
16	[redacted] (15)	-	109	67	82	86	21
17	[redacted] (5)	-	108	94	94	99	8
18	Purified water (0.1 ml)	-	118	107	96	107	11
19	Benzo[a]pyrene (5)	+	412	377	426	405	25
20	ENNG (3)	-	370	386	413	390	22
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	135	130	130	132	3
sd	Standard deviation						

TABLE 2 - continued

Results obtained with *Salmonella typhimurium* TA100 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means Test 2 (with pre-incubation) A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted] (5000); sterility check	-	0	0	0	0	0
3	[redacted] (5000)	+	99	94	107	100	7
4	[redacted] (1500)	+	97	101	99	99	2
5	[redacted] (500)	+	99	97	102	99	3
6	[redacted] (150)	+	87	106	102	98	10
7	[redacted] (50)	+	85	94	112	97	14
8	Purified water (0.1 ml)	+	104	115	89	103	13
9	[redacted] (5000)	-	109	104	89	101	10
10	[redacted] (1500)	-	100	99	108	102	5
11	[redacted] (500)	-	95	88	96	93	4
12	[redacted] (150)	-	95	109	102	102	7
13	[redacted] (50)	-	95	90	88	91	4
14	Purified water (0.1 ml)	-	95	103	92	97	6
15	Benzo[a]pyrene (5)	+	391	480	414	428	46
16	ENNG (3)	-	384	387	395	389	6
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	106	107	107	107	1
sd	Standard deviation						

TABLE 3

Results obtained with *Salmonella typhimurium* TA1535 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 1				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted](5000); sterility check	-	0	0	0	0	0
3	[redacted](5000)	+	19	16	17	17	2
4	[redacted](1500)	+	15	20	23	19	4
5	[redacted]500)	+	15	19	16	17	2
6	[redacted](150)	+	22	16	16	18	3
7	[redacted](50)	+	14	12	14	13	1
8	[redacted](15)	+	21	17	16	18	3
9	[redacted](5)	+	21	20	21	21	1
10	Purified water (0.1 ml)	+	16	17	23	19	4
11	[redacted]5000)	-	12	19	27	19	8
12	[redacted]1500)	-	20	12	15	16	4
13	[redacted]500)	-	19	28	16	21	6
14	[redacted](150)	-	15	17	15	16	1
15	[redacted](50)	-	19	15	9	14	5
16	[redacted](15)	-	19	21	34	25	8
17	[redacted](5)	-	19	19	15	18	2
18	Purified water (0.1 ml)	-	17	22	19	19	3
19	2-Aminoanthracene (2)	+	129	119	132	127	7
20	ENNG (5)	-	100	117	129	115	15
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	141	129	139	136	6
sd	Standard deviation						

TABLE 3 - continued

Results obtained with *Salmonella typhimurium* TA1535 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means Test 2 (with pre-incubation) A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted]5000); sterility check	-	0	0	0	0	0
[redacted]	[redacted](5000)	+	20	14	14	16	3
[redacted]	[redacted](1500)	+	20	21	20	20	1
5	[redacted](500)	+	21	14	14	16	4
6	[redacted](150)	+	22	22	14	19	5
7	[redacted](50)	+	21	14	20	18	4
8	Purified water (0.1 ml)	+	20	23	19	21	2
9	[redacted]5000)	-	16	21	13	17	4
10	[redacted]1500)	-	14	13	19	15	3
11	[redacted](500)	-	23	23	22	23	1
12	[redacted](150)	-	20	22	14	19	4
13	[redacted]50)	-	20	20	23	21	2
14	Purified water (0.1 ml)	-	17	22	19	19	3
15	2-Aminoanthracene (2)	+	125	118	112	118	7
16	ENNG (5)	-	90	123	117	110	18
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	121	111	117	116	5
sd	Standard deviation						

TABLE 4

Results obtained with *Salmonella typhimurium* TA1537 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 1				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted] (5000); sterility check	-	0	0	0	0	0
3	[redacted] (5000)	+	8	8	10	9	1
4	[redacted] (1500)	+	9	10	12	10	2
5	[redacted] (500)	+	10	13	12	12	2
6	[redacted] (150)	+	12	12	9	11	2
7	[redacted] (50)	+	15	10	10	12	3
8	[redacted] (15)	+	10	15	9	11	3
9	[redacted] (5)	+	13	8	8	10	3
10	Purified water (0.1 ml)	+	12	12	10	11	1
11	[redacted] (5000)	-	15	14	13	14	1
12	[redacted] (1500)	-	16	10	16	14	3
13	[redacted] (500)	-	12	12	9	11	2
14	[redacted] (150)	-	12	13	13	13	1
15	[redacted] (50)	-	10	12	10	11	1
16	[redacted] (15)	-	10	12	6	9	3
17	[redacted] (5)	-	13	9	12	11	2
18	Purified water (0.1 ml)	-	10	14	13	12	2
19	Benzo[a]pyrene (5)	+	206	237	202	215	19
20	9-Aminoacridine (30)	-	245	189	202	212	29
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	115	121	119	118	3
sd	Standard deviation						

TABLE 4 - continued

Results obtained with *Salmonella typhimurium* TA1537 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means Test 2 (with pre-incubation) A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted](5000); sterility check	-	0	0	0	0	0
3	[redacted](5000)	+	9	12	10	10	2
4	[redacted](1500)	+	10	16	9	12	4
5	[redacted](500)	+	6	10	12	9	3
6	[redacted](150)	+	9	8	15	11	4
7	[redacted](50)	+	12	14	12	13	1
8	Purified water (0.1 ml)	+	7	8	12	9	3
9	[redacted](5000)	-	12	8	7	9	3
10	[redacted](1500)	-	8	12	8	9	2
11	[redacted](500)	-	8	14	8	10	3
12	[redacted](150)	-	12	13	12	12	1
13	[redacted](50)	-	10	7	12	10	3
14	Purified water (0.1 ml)	-	10	7	10	9	2
15	Benzo[a]pyrene (5)	+	174	176	203	184	16
16	9-Aminoacridine (30)	-	143	141	136	140	4
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	93	115	108	105	11
sd	Standard deviation						

TABLE 5

Results obtained with *Escherichia coli* CM891 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 1				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted] (5000); sterility check	-	0	0	0	0	0
3	[redacted] (5000)	+	96	99	99	98	2
4	[redacted] (1500)	+	100	111	94	102	9
5	[redacted] (500)	+	119	108	90	106	15
6	[redacted] (150)	+	108	107	99	105	5
7	[redacted] (50)	+	106	96	97	100	6
8	[redacted] (15)	+	100	102	103	102	2
9	[redacted] (5)	+	115	101	95	104	10
10	Purified water (0.1 ml)	+	96	107	110	104	7
11	[redacted] (5000)	-	102	100	109	104	5
12	[redacted] (1500)	-	119	108	86	104	17
13	[redacted] (500)	-	108	109	100	106	5
14	[redacted] (150)	-	99	87	92	93	6
15	[redacted] (50)	-	81	112	109	101	17
16	[redacted] (15)	-	104	93	88	95	8
17	[redacted] (5)	-	110	88	96	98	11
18	Purified water (0.1 ml)	-	107	95	112	105	9
19	2-Aminoanthracene (10)	+	435	474	415	441	30
20	ENNG (2)	-	768	758	740	755	14
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	123	110	117	117	7
sd	Standard deviation						

TABLE 5 - continued

Results obtained with *Escherichia coli* CM891 following exposure to [redacted]

Plate No.	Addition (µg)	S9 mix + present - absent	Revertant colony counts and means				
			Test 2 (with pre-incubation)				
			A	B	C	Mean	sd
1	None; sterility check	+	0	0	0	0	0
2	[redacted]5000); sterility check	-	0	0	0	0	0
3	[redacted]5000)	+	87	96	96	93	5
4	[redacted]1500)	+	100	93	103	99	5
5	[redacted]500)	+	94	96	101	97	4
6	[redacted]150)	+	115	125	114	118	6
7	[redacted]50)	+	102	114	115	110	7
8	Purified water (0.1 ml)	+	102	100	109	104	5
9	[redacted]5000)	-	102	100	78	93	13
10	[redacted]1500)	-	107	118	128	118	11
11	[redacted]500)	-	101	90	107	99	9
12	[redacted]150)	-	97	104	101	101	4
13	[redacted]50)	-	115	102	114	110	7
14	Purified water (0.1 ml)	-	112	131	93	112	19
15	2-Aminoanthracene (10)	+	443	402	465	437	32
16	ENNG (2)	-	842	886	900	876	30
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar (total counts)	-	123	124	111	119	7
sd	Standard deviation						

APPENDIX 1

Historical control data

Presented below are the historical control data from the period 1 April 1997 to 30 June 1998.

Purified water solvent controls

Strain	TA100		TA1535		CM891		TA98		TA1537	
S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	78	81	12	10	79	73	26	27	7	7
Maximum	122	123	33	29	164	211	45	47	21	22
Mean	99.5	100.4	18.7	18.8	112.0	121.3	36.1	38.1	12.1	12.2
No. of values	99	99	95	95	31	31	98	98	96	96

Positive controls

Strain	TA100		TA1535		CM891		TA98		TA1537		
S9 mix	-	+	-	+	-	+	-	+	- (80 µg)	- (30 µg)	+
Minimum	190	240	37	80	294	231	117	200	562	169	94
Maximum	1477	1231	1243	533	2312	2097	649	823	4532	384	396
Mean	377.3	510.8	193.8	233.3	1296.7	723.1	243.7	488.8	1695.4	251.5	210.2
No. of values	274	273	268	267	89	89	271	270	266	6	271