IQB-9302

BACTERIAL MUTATION ASSAY

IQB-9302

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Sponsor

Laboratorios INIBSA, SA,
Crta. Sabadell a Granollers, Km.14.5,
08185 Lliça de Vall,
Barcelona,
SPAIN.

Research Laboratory

Huntingdon Life Sciences Ltd.,
P.O. Box 2,
Huntingdon,
Cambridgeshire,
PE18 6ES,
ENGLAND.

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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 and I consider the data generated to be valid.

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

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

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

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Jonathan Kitching, B.Sc. (Hons.),
Study Director,
Department of Genetic Toxicology,
Huntingdon Life Sciences Ltd..

17 February 1999
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 review	6 January 1999	6 January 1999
Process Based Inspections		
Preparation & labelling Agar preparation	} 20 November 1998	} 9 December 1998
Test material preparation Dosing	} 20 November 1998	} 9 December 1998
Plate counting	8 December 1998	9 December 1998
Record keeping	9 December 1998	9 December 1998
S9 fraction preparation	9 November 1998	10 November 1998
Report	28 January 1999	29 January 1999

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.

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Margaret Blows, Date
Quality Assurance Group Leader,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

SUMMARY

In this *in vitro* assessment of the mutagenic potential of IQB-9302, histidine dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA1535, TA1537, TA98 and TA100) and a tryptophan dependent mutant of *Escherichia coli* (strain CM891) were exposed to the test substance diluted in 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.

Dose levels of up to 5000 μg/plate were tested in the mutation tests. This is the standard limit dose recommended in the regulatory guidelines this assay follows. Other dose levels 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 dose level of IQB-9302 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 water, IQB-9302 shows no evidence of mutagenic activity in this bacterial system.

INTRODUCTION

This report describes a study designed to assess the mutagenic potential of IQB-9302 in a bacterial system. The study was conducted in compliance with the following guidelines:

> OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guidelines 471.
>
> 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.

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 8 October 1998, the Sponsor

on 20 October 1998 and by the Study Director on 18 December 1998.

The experimental phase of the study was conducted between 8 and 15 January 1999.

TEST SUBSTANCE

Identity:	IQB-9302.HCl
Chemical name:	dl-1-(Methylcyclopropyl)-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide, hydrochloride; dl-1-(Methylcyclopropyl-2',6'-pipecoloxylidide, hydrochloride
Appearance:	White crystalline powder
Storage conditions:	Room temperature in the dark
Batch number:	9454.001
Expiry date:	12 months from date of receipt
Purity:	101.0%
Date received:	28 October 1998

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The following strains were used:-

S. typhimurium TA1535 *his*G46 *rfa uvr*B

S. typhimurium TA1537 *his*C3076 *rfa uvr*B

S. typhimurium TA98 *his*D3052 *rfa uvr*B pKM101

S. typhimurium TA100 *his*G46 *rfa uvr*B pKM101

E. coli CM891 WP2 *trp uvr*A pKM101

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 -80ºC. 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 were 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 2×10^9 cells per ml which were measured photometrically.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	*N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine
Supplier:	Sigma Chemical
Batch number:	67F-3700
Appearance:	Pale yellow crystalline powder
Solvent:	DMSO
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:	35H1408
Appearance:	Yellow powder
Solvent:	DMSO
Concentration:	30 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
Supplier:	Aldrich Chemical Company
Batch number:	75108
Appearance:	Beige powder
Solvent:	DMSO
Concentration:	1 µg/plate for strain TA98

In the presence of S9 mix

Identity:	2-Aminoanthracene
Supplier:	Aldrich Chemical Company
Batch number:	0013406
Appearance:	Green powder
Solvent:	DMSO
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain CM891

Identity:	Benzo[a]pyrene
Supplier:	Aldrich Chemical Company
Batch number:	05915DY
Appearance:	Yellow powder
Solvent:	DMSO
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
Diet:	SDS R+M no.1 (modified) nuts

S9 fraction was prepared from a group of *ca* 20 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.

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

Prior to commencing testing, the solubility of the test substance was assessed at 50 mg/ml in water, in which it dissolved with *ca* 2 minutes whirlimixing. Therefore water was used as the solvent for this study.

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 IQB-9302 tested was 50 mg/ml in the chosen solvent, which provided a final concentration of 5000 µg/plate. This is the standard limit dose recommended in the regulatory guidelines this assay follows. The negative control was the chosen solvent, 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 bottles. An aliquot of 100 µl of the test solution was added, followed immediately by 2 ml of histidine/tryptophan deficient agar. 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 dose level. 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 Seescan 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 bottles 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 dose levels were used as it was known from the first test that the test substance was non-toxic.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and 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 be in the range stated in the appropriate Standard Operating Procedure. These ranges are based on the laboratory's historical control values. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the solvent control.

The mean number of revertant colonies for all treatment groups is compared with those obtained for the solvent control groups. The mutagenic activity of a test substance is 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. Modifications to the experimental method will usually be considered, such as the use of a narrower dose range and different levels of S9 in the mix. 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.

RESULTS

The mean number of revertant colonies, together with the individual plate counts for IQB-9302 obtained in the first mutation test with the tester strains are shown in Table 1. Positive control mutability checks are shown in Table 2. No toxicity was observed towards the tester strains.

The mean number of revertant colonies, together with the individual plate counts for IQB-9302 obtained in the second mutation test with the tester strains are shown in Table 3. Positive control mutability checks are shown in Table 4.

No substantial increases in revertant colony numbers of any of the tester strains were observed following treatment with IQB-9302 at any dose level, in the presence or absence of S9 mix, in either mutation test.

The mean revertant colony counts for the solvent controls were within the historical range. The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations by causing increases over double the concurrent solvent control.

CONCLUSION

It is concluded that, when tested in water, IQB-9302 shows no evidence of mutagenic activity in this bacterial system.

REFERENCES

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TABLE 1

Mutation Test 1

IQB-9302 - revertant colony counts obtained per plate using bacterial strains TA1535, TA1537 and TA98

Strain	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA1535	5000	-	14	4.0	18, 10, 14
	1500	-	18	4.7	16, 23, 14
	500	-	13	3.6	17, 12, 10
	150	-	16	3.8	19, 12, 18
	50	-	15	5.5	21, 12, 11
	15	-	16	2.1	18, 14, 17
	5	-	19	3.1	22, 16, 18
	Solvent	-	19	2.5	19, 17, 22
	5000	+	15	2.0	15, 13, 17
	1500	+	19	2.5	17, 22, 19
	500	+	14	2.6	13, 12, 17
	150	+	18	1.5	16, 19, 18
	50	+	13	1.0	13, 12, 14
	15	+	13	2.5	11, 16, 13
	5	+	18	1.2	17, 19, 19
	Solvent	+	17	0.6	17, 17, 16
TA1537	5000	-	13	4.4	11, 18, 10
	1500	-	11	3.1	14, 8, 10
	500	-	9	2.6	6, 10, 11
	150	-	8	4.0	13, 6, 6
	50	-	13	3.8	11, 10, 17
	15	-	12	2.0	10, 12, 14
	5	-	10	0.6	9, 10, 10
	Solvent	-	14	5.3	16, 8, 18
	5000	+	8	2.1	6, 7, 10
	1500	+	13	2.1	11, 15, 12
	500	+	10	2.1	8, 12, 11
	150	+	11	2.1	13, 9, 10
	50	+	11	2.5	14, 9, 11
	15	+	9	4.4	7, 6, 14
	5	+	9	1.2	10, 8, 8
	Solvent	+	16	4.2	19, 11, 17
TA98	5000	-	26	2.3	25, 25, 29
	1500	-	21	3.6	18, 25, 20
	500	-	23	4.6	26, 26, 18
	150	-	26	5.1	25, 22, 32
	50	-	22	2.1	20, 21, 24
	15	-	22	3.0	19, 25, 22
	5	-	23	2.6	22, 26, 21
	Solvent	-	23	1.5	22, 23, 25
	5000	+	29	2.1	27, 31, 28
	1500	+	30	6.9	34, 22, 34
	500	+	32	1.2	31, 31, 33
	150	+	28	6.0	34, 29, 22
	50	+	28	4.9	25, 26, 34
	15	+	29	7.6	22, 27, 37
	5	+	28	5.0	33, 23, 27
	Solvent	+	30	4.6	31, 25, 34

– Absence
\+ Presence
SD Standard deviation

TABLE 1

(continued)

Mutation Test 1

IQB-9302 - revertant colony counts obtained per plate using bacterial strains TA100 and CM891

Strain	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA100	5000	-	115	13.3	123, 100, 123
	1500	-	133	4.0	137, 132, 129
	500	-	138	6.7	146, 134, 135
	150	-	138	14.7	154, 125, 135
	50	-	144	18.5	125, 144, 162
	15	-	159	12.6	146, 171, 161
	5	-	135	13.3	143, 120, 143
	Solvent	-	151	7.0	144, 150, 158
	5000	+	133	7.2	125, 137, 138
	1500	+	112	8.4	107, 122, 108
	500	+	128	11.2	124, 120, 141
	150	+	145	11.1	147, 133, 155
	50	+	139	12.7	134, 153, 129
	15	+	144	11.4	131, 147, 153
	5	+	121	16.7	116, 140, 108
	Solvent	+	132	7.5	140, 132, 125
CM891	5000	-	85	9.5	94, 86, 75
	1500	-	117	9.8	106, 125, 120
	500	-	170	12.7	160, 165, 184
	150	-	198	3.6	201, 194, 199
	50	-	158	14.2	174, 147, 153
	15	-	191	9.1	201, 190, 183
	5	-	159	7.9	162, 165, 150
	Solvent	-	180	18.9	167, 202, 172
	5000	+	101	20.6	115, 110, 77
	1500	+	135	18.9	151, 139, 114
	500	+	208	24.8	212, 230, 181
	150	+	226	9.5	219, 237, 223
	50	+	209	22.9	235, 197, 194
	15	+	214	14.1	229, 212, 201
	5	+	195	6.1	202, 191, 192
	Solvent	+	207	20.6	199, 191, 230

– Absence
\+ Presence
SD Standard deviation

TABLE 2

Mutation Test 1

Revertant colony counts obtained per plate with positive controls

Strain	Compound	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA1535	ENNG	5	-	888	18.5	889, 906, 869
TA1537	9 AC	30	-	154	20.1	140, 177, 145
TA98	NF	1	-	147	2.0	147, 145, 149
TA100	ENNG	3	-	701	15.1	717, 699, 687
CM891	ENNG	2	-	2071	88.0	2122, 2121, 1969
TA1535	AA	2	+	192	2.3	193, 189, 193
TA1537	B[a]P	5	+	79	12.2	82, 66, 90
TA98	B[a]P	5	+	267	16.8	286, 256, 258
TA100	B[a]P	5	+	736	47.7	727, 788, 694
CM891	AA	10	+	2022	42.9	2012, 2069, 1985

– Absence
\+ Presence
SD Standard deviation
ENNG *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine
9 AC 9-Aminoacridine
NF 2-Nitrofluorene
AA 2-Aminoanthracene
B[a]P Benzo[a]pyrene

TABLE 3

Mutation Test 2

IQB-9302 - revertant colony counts obtained per plate using bacterial strains TA1535, TA1537 and TA98

Strain	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA1535	5000	-	12	3.1	15, 9, 11
	1500	-	14	6.1	11, 10, 21
	500	-	18	0.0	18, 18, 18
	150	-	16	3.0	19, 16, 13
	50	-	15	3.6	18, 11, 16
	Solvent	-	17	3.6	20, 13, 18
	5000	+	14	2.1	12, 13, 16
	1500	+	14	2.6	15, 16, 11
	500	+	11	1.5	9, 12, 11
	150	+	14	2.3	11, 15, 15
	50	+	15	5.0	20, 16, 10
	Solvent	+	17	2.5	20, 17, 15
TA1537	5000	-	11	1.7	10, 13, 10
	1500	-	12	1.0	11, 13, 12
	500	-	9	3.8	13, 6, 7
	150	-	9	1.0	9, 10, 8
	50	-	10	0.6	11, 10, 10
	Solvent	-	12	2.1	14, 13, 10
	5000	+	13	5.6	7, 14, 18
	1500	+	15	4.6	18, 18, 10
	500	+	10	0.6	10, 11, 10
	150	+	12	1.2	11, 13, 13
	50	+	12	2.5	10, 15, 12
	Solvent	+	15	2.5	12, 17, 15
TA98	5000	-	23	3.5	23, 20, 27
	1500	-	23	2.6	21, 26, 22
	500	-	24	2.1	26, 23, 22
	150	-	24	0.6	24, 25, 24
	50	-	25	0.6	25, 25, 24
	Solvent	-	26	6.4	22, 33, 22
	5000	+	23	3.1	24, 26, 20
	1500	+	31	4.9	29, 28, 37
	500	+	24	2.5	24, 26, 21
	150	+	30	3.8	34, 27, 28
	50	+	20	3.1	17, 21, 23
	Solvent	+	26	3.2	22, 27, 28

– Absence
\+ Presence
SD Standard deviation

TABLE 3

(continued)

Mutation Test 2

IQB-9302 - revertant colony counts obtained per plate using bacterial strains TA100 and CM891

Strain	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA100	5000	-	105	12.7	120, 98, 98
	1500	-	108	7.0	103, 116, 105
	500	-	113	5.1	107, 114, 117
	150	-	112	15.4	105, 130, 102
	50	-	113	9.8	102, 116, 121
	Solvent	-	132	13.5	133, 145, 118
	5000	+	104	10.6	108, 92, 112
	1500	+	91	12.3	100, 96, 77
	500	+	83	18.0	82, 65, 101
	150	+	81	8.6	79, 73, 90
	50	+	78	7.8	69, 82, 83
	Solvent	+	75	3.5	78, 75, 71
CM891	5000	-	185	12.7	170, 191, 193
	1500	-	208	4.0	209, 204, 212
	500	-	198	1.5	198, 197, 200
	150	-	206	4.9	203, 204, 212
	50	-	201	12.9	190, 215, 197
	Solvent	-	189	12.3	180, 203, 184
	5000	+	184	25.4	166, 173, 213
	1500	+	207	25.0	208, 231, 181
	500	+	249	17.7	229, 258, 261
	150	+	201	19.3	223, 186, 195
	50	+	198	19.2	185, 189, 220
	Solvent	+	215	13.1	229, 214, 203

– Absence
\+ Presence
SD Standard deviation

TABLE 4

Mutation Test 2

Revertant colony counts obtained per plate with positive controls

Strain	Compound	Dose level (µg/plate)	Liver S9 mix	Mean revertant colony counts	SD	Individual revertant colony counts
TA1535	ENNG	5	-	1220	15.5	1204, 1220, 1235
TA1537	9 AC	30	-	745	56.3	713, 712, 810
TA98	NF	1	-	166	1.2	167, 165, 165
TA100	ENNG	3	-	658	36.9	700, 641, 632
CM891	ENNG	2	-	2058	14.7	2066, 2067, 2041
TA1535	AA	2	+	171	8.5	165, 181, 168
TA1537	B[a]P	5	+	78	9.5	79, 87, 68
TA98	B[a]P	5	+	305	0.0	305, 305, 305
TA100	B[a]P	5	+	471	18.6	476, 486, 450
CM891	AA	10	+	1962	37.0	1963, 1999, 1925

– Absence
\+ Presence
SD Standard deviation
ENNG *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine
9 AC 9-Aminoacridine
NF 2-Nitrofluorene
AA 2-Aminoanthracene
B[a]P Benzo[a]pyrene

APPENDIX 1

Historical control data

Presented below are the historical control data from the five years prior to commencing this study.

Water solvent controls

Strain	TA1535		TA1537		TA98		TA100		CM891	
Liver S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	9	10	4	3	14	18	71	71	169	150
Maximum	21	22	18	19	34	53	160	164	330	340
Mean	15.0	16.5	10.5	11.4	23.7	26.6	121.7	126.3	240.8	228.8
SD	2.4	2.4	2.4	2.5	3.5	3.2	14.9	15.2	43.6	45.1
Min (99% limit)	9	10	4	5	15	18	83	87	129	113
Max (99% limit)	21	23	17	18	33	35	160	165	353	345
No. of Values	249	235	246	233	253	243	258	248	41	40

Positive controls

Strain	TA1535		TA1537		TA98		TA100		CM891	
Liver S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	68	31	42	24	69	53	149	184	620	381
Maximum	2568	451	3500	714	1260	1617	1984	2498	3500	2192
Mean	390.2	145.0	3204.4	79.0	267.0	170.4	461.1	430.8	1518.5	1342.2
SD	341.4	56.8	922.9	52.0	106.5	113.5	184.5	189.8	425.7	476.5
No. of Values	780	769	777	771	779	778	791	786	146	146

APPENDIX 2

Certificate of analysis