RESEARCH TOXICOLOGY CENTRE - ROMA

[redacted]

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

FINAL REPORT

RTC Report No.: 8837-001-M-06001

Sponsor:
AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

RTC Report No.: 8837-001-M-06001

COMPLIANCE STATEMENT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted, and the results obtained in the performance of the study. The aspects of the study conducted by Research Toxicology Centre S.p.A. were performed in accordance with:

A. *"Good Laboratory Practice Regulations"* of the U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the principles of good laboratory practice as specified in Council Directive 87/18/EEC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (adoption of the *"OECD principles on Good Laboratory Practice – as revised in 1997"*) and subsequent revisions.

C. Decreto Legislativo 27 gennaio 1992, n. 120 published in the Gazzetta Ufficiale della Repubblica Italiana 18 Febbraio 1992 (adoption of the Commission Directive of 18 December 1989 adapting to technical progress the Annex to Council Directive 88/320/EEC on the inspection and verification of Good Laboratory Practice (90/18/EEC)) and subsequent revisions.

Orietta Scarcella 07 Nov 2001

O. Scarcella, Biol.D.
(Study Director) : Date :

J. Brightwell, Ph.D.
(Scientific Director) : Date : 7.11.2001

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QUALITY ASSURANCE STATEMENT
(Relevant to the aspects of the study conducted by RTC)

Study phases monitored by RTC's QAU according to current relevant Standard Operating Procedures	Quality Assurance Inspections (Day Month Year)		
	Inspection	Report to Study Director	Report to Company Management
PROTOCOL CHECK	08.08.2001	09.08.2001	09.08.2001
PROCESS-BASED INSPECTIONS			
Dose preparation	18.09.2001	-	26.09.2001
Treatment	18.09.2001	-	03.10.2001
Plating out	18.09.2001	-	03.10.2001
Plate scoring	09.07.2001	-	12.07.2001
Other routine inspections of a procedural nature were carried out on activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.			
FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.	Review completed 06/Nov 2001		

M.M. Brunetti, Biol.D.
(Head of Quality Assurance)

06/Nov/2001
Date

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Contents

1. SUMMARY

1.1 The test item [redacted] was examined for the ability to induce gene mutations in tester strains of *Salmonella typhimurium* and *Escherichia coli,* as measured by reversion of auxotrophic strains to prototrophy. The five tester strains TA1535, TA1537, TA98, TA100 and WP2 *uvr*A were used. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.
Test item solutions were prepared using dimethylsulphoxide.

1.2 In the toxicity test, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. In the absence of S9 metabolism toxicity was observed at the highest dose-level with all tester strains, while slight toxicity was observed with TA1537 at the same dose level in the presence of metabolic activation.

1.3 Two main experiments were performed.
In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate. Microcolony formation was observed at the highest dose-level with TA1537, in the absence of S9 metabolism.
As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step. The same dose-range employed in Main Assay I was used with all tester strains with the exception of TA1537 which was treated, in the absence of S9 metabolism, at the five dose-levels ranging from 2500 to 156 µg/plate. Toxicity was observed at the highest or at the two higher dose-levels tested with all tester strains.

1.4 The test item did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

1.5 It is concluded that the test item [redacted] does not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item to *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and to *Escherichia coli* strain WP2 *uvr*A using the procedures developed by Ames *et al.*, 1975 and revised by Maron and Ames, 1983.

In addition, the study was designed to comply with the experimental methods indicated in:

- EEC Council Directive 2000/32, Annex 4D.
- OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997).

2.2 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2.3 Study organisation

Sponsor:

AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

Location of Study:

Genetic Toxicology Department
Research Toxicology Centre S.p.A.
Via Tito Speri, 12
00040 Pomezia (Roma)
Italy

Principal dates:

Study protocol approved by Study Director: 20-Jul-2001
Study commenced: 25-Sept-2001 (Toxicity assay treatment)
Study completed: 08-Oct-2001 (Completion of scoring Main Assay II)

Study Director:

O. Scarcella, Biol.D.

Archiving:

The original data arising from this study and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of at least five years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material, or further archiving. An aliquot of the test item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

3. MATERIALS AND METHODS

3.1 Test item

A 120 g sample of [redacted], Lot No.: 90199/66, was received from AUSIMONT S.p.A. on 14-May-2001 and was stored within the Formulation Unit at RTC at room temperature. The test item, a colourless liquid, was contained in an opaque plastic bottle. Information received from the Sponsor indicates that the test item is a Dicarboxy derivative of perfluoropolyoxyalkane at a concentration >99%.
On 24-Sept-2001 a 2 g sub-sample was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions.

Solutions of the test item, as received, were prepared immediately before use in dimethylsulphoxide. Solutions were prepared on a weight/volume basis without correction for the displacement due to the volume of the test item. Concentrations were expressed in terms of active constituent. All test item solutions were used within 2 hours and 45 minutes of the initial formulation. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose-levels in this report are expressed to three significant figures.

3.2 Control items

The solvent used in this study was dimethylsulphoxide (DMSO; Fluka AG, batch 404161/1 13400).

Positive control treatments used solutions prepared as follows:

Sodium azide (Fluka AG, batch 221999 1081) in distilled water.
9-Aminoacridine (ICN K&K Laboratories, batch 12058-A) in DMSO.
2-Nitrofluorene (EGA Chemie, batch 12532) in DMSO.
2-Aminoanthracene (Sigma, batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (Fluka AG, batch 359316/153696) in distilled water.
Sterile distilled water (BIEFFE, batch 01C0201)

3.3 Media

The following growth media were used:

Nutrient Broth: Oxoid Nutrient Broth No 2 was prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This was used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) were added to distilled water (1 litre) and autoclaved.
The solutions were then poured into plastic Petri dishes and allowed to solidify and dry before use. These plates were used for the non-selective growth of the tester strains.

Minimal Agar: Minimal medium agar was prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) was prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) was added to the top agar (100 ml).

3.4 S9 tissue homogenate

Two batches of S9 tissue homogenate (designated 2001/10 and 2001/12) were used in this study and had the following characteristics:

S9 Batch	Protein content (mg/ml)	Aminopyrine demethylase activity (µM/g liver/5 min, formaldehyde production)
2001/10	32.0 ± 3.13	3.62 ± 0.14
2001/12	32.3 ± 1.96	3.66 ± 0.11

Each S9 tissue fraction was prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes. The efficacy of the S9 tissue fraction was previously checked in an Ames test and produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.5 Bacterial strains

Four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and a strain of *Escherichia coli* (WP2 *uvr*A) were used in this study. Permanent stocks of these strains are kept at -80°C in RTC. Overnight subcultures of these stocks were prepared for each day's work.
Bacteria were taken from vials of frozen cultures, which had been checked for the presence of the appropriate genetic markers, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Bacterial cultures in liquid and on agar were clearly identified with their identity.

3.6 Methods

3.6.1 Preliminary toxicity test

A preliminary toxicity test was undertaken in order to select the concentrations of the test item to be used in the main assays. In this test a wide range of dose-levels of the test item, set at half-log intervals, were used. Treatments were performed both in the absence and presence of S9 metabolism using the plate incorporation method; a single plate was used at each test point and positive controls were not included.
Toxicity was assessed on the basis of a decline in the number of spontaneous revertants, a thinning of the background lawn or a microcolony formation.

3.6.2 Main experiments

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolising system. Three replicate plates were used at each test point.

In addition, plates were prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures were plated on nutrient agar plates to establish the number of bacteria in the cultures.

The first experiment was performed using a plate-incorporation method. The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture was composed as follows:

(i)	Overlay agar (held at 45°C)	2	ml
(ii)	Test or control item solution	0.1	ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5	ml
(iv)	Bacterial suspension	0.1	ml

The second experiment was performed using a pre-incubation method. The components were added in turn to an empty test-tube:

(i)	Bacterial suspension	0.1	ml
(ii)	Test item or control item solution	0.05	ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5	ml

The incubate was vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

3.6.3 Incubation and scoring

The prepared plates were inverted and incubated for approximately 72 hours at 37°C. After this period of incubation, the scoring was effected by counting the number of revertant colonies on each plate.

4. RESULTS

4.1 Solubility

The test item was found to be soluble in dimethylsulphoxide at a concentration of 100 mg/ml . Since 100 µl of the test item solution are used in the preparation of each plate, this permitted a maximum concentration of 5000 µg/plate to be used in the toxicity test.

4.2 Toxicity test

The test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Results are presented in Tables 1 and 2.

In the absence of S9 metabolic activation, toxicity, as indicated by reduction in revertant numbers and/or thinning of the background lawn was observed at the highest dose-level with all tester strains. In the presence of S9 metabolism, slight toxicity was observed at 5000 µg/plate with TA1537. On the basis of these results a maximum concentration of 5000 µg/plate was selected for the Main Assay with all tester strains.

4.3 Assay for reverse mutation

Two experiments were performed; individual plate counts for these tests, and the mean and standard error of the mean for each test point, together with statistical analysis are presented in Tables 3 to 12.

In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels, separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate. Toxicity, as indicated by reduction in revertant numbers and/or thinning of the background lawn was observed at the highest dose-level with all tester strains in the absence of S9 metabolism. A more pronounced effect, as indicated by microcolony formation, was noted in TA1537 tester strain. In the presence of S9 metabolism just slight toxicity was evident at 5000 µg/plate with TA1537.

As no increases in revertant numbers were observed, all treatments of Main Assay II included a pre-incubation step and used the same dose-range employed in Main Assay I with all tester strains with the exception of TA1537. With this tester strain, in the absence of S9 metabolism, the test item was assayed at the five dose-levels of 2500, 1250, 625, 313 and 156 µg/plate. Toxicity was observed in all tester strains at the highest or at the two higher dose-levels both in the absence and presence of S9 metabolism.

The test item did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism.

The sterility of the S9 mix and the test item solutions was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in these tests following treatment with the positive control items, indicating that the assay system was functioning correctly.

5. ANALYSIS OF RESULTS

5.1 Criteria for outcome of the assays

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition, there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

5.2 Evaluation

The test item does not induce increases in the number of revertant colonies, at any dose-level, in any tester strain, in the absence or presence of S9 metabolism. On the basis of the stated criteria it must be concluded that the test item [redacted] is not mutagenic to *S. typhimurium* and *E. coli* under the reported experimental conditions.

6. CONCLUSION

It is concluded that the test item [redacted] does not induce reverse mutation in *S. typhimurium* and *E. coli* under the reported experimental conditions.

7. KEY TO TABLES 1-12

7.1 Structure of Tables 3-12

These tables show, for each *Salmonella typhimurium* or *Escherichia coli* tester strain, the individual plate counts obtained for the negative and positive controls, and at each dose-level of the test item. The mean number of revertant colonies and standard error of the mean are also presented. The "untreated" plates receive no treatment, while the plates at dose-level 0.00 are solvent control plates. The titre of the bacterial cultures is given (million cells/plate).

7.2 Regression line

i) The regression analysis fits a regression line to the data by the least squares method, after square root transformation of the plate counts to satisfy normal distribution and homoscedasticity assumptions. The regression equation is expressed as:

$y = a + bx$

where y = transformed revertant numbers
a = intercept
b = slope value
x = dose-level (in the units given).

ii) The regression line includes the solvent control data but not the untreated control data.

iii) Regression lines are calculated using a minimum of the three lowest dose-levels, and then including the further dose-levels in turn. The correlation co-efficient (r), the value of students "t" statistic, and the p-value for the regression lines are also given.

8. TABLES 1 TO 12

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[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 1 - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 8837-001

SOLVENT: DMSO

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 uvrA Rev/pl.
Untreated	24	20	34	143	32
0.00	22	19	38	138	30
50.0	15	21	31	135	35
158	23	18	38	140	31
500	14	20	37	128	27
1580	16	19	30	114	23
5000	10 *	11 *	27 *	97 *	21 *

* = thinning of the background lawn

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[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 2 - WITH METABOLIC ACTIVATION

STUDY NO.: 8837-001

SOLVENT: DMSO

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 uvrA Rev/pl.
Untreated	17	21	45	160	40
0.00	15	22	39	156	41
50.0	14	20	38	172	42
158	19	21	42	181	40
500	18	19	36	208	40
1580	14	16	37	178	31
5000	13	18 *	38	182	34

* = thinning of the background lawn

REDACTED AS TO TRADE NAMES

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[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 3 - Experiment I - Plate incorporation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA1535 Titre: 224

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	21	19	21		20	0.7	14	17	16	16	0.9
0.00	23	22	24		23	0.6	18	20	19	19	0.6
313	17	24	18		20	2.2	12	15	19	15	2.0
625	15	15	21		17	2.0	18	19	23	20	1.5
1250	16	19	22		19	1.7	17	13	17	16	1.3
2500	18	18	23		20	1.7	19	22	22	21	1.0
5000	14	17	20	*	17	1.7	10	13	15	13	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.785	-0.0011	-0.70631	2.6398	0.03343
1 - 4	-	4.598	-0.0003	-0.40465	1.3993	0.19197
1 - 5	-	4.485	-0.0001	-0.17059	0.6242	0.54327
1 - 6	-	4.489	-0.0001	-0.35009	1.4950	0.15439
1 - 3	+	4.187	0.0002	0.12303	0.3280	0.75250
1 - 4	+	4.277	-0.0002	-0.25995	0.8513	0.41452
1 - 5	+	4.137	0.0001	0.29370	1.1078	0.28802
1 - 6	+	4.308	-0.0001	-0.42152	1.8594	0.08147

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	21	19	21	20	0.7
Sodium Azide	1 µg/pl	-	581	617	633	610	15.4
DMSO	100 µl/pl	+	18	20	19	19	0.6
2-Aminoanthracene	1 µg/pl	+	106	132	125	121	7.8

* = thinning of the background lawn

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[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 4 - Experiment I - Plate incorporation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA1537 Titre: 218

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	19	19	20		19	0.3	18	19	22		20	1.2
0.00	15	21	20		19	1.9	21	25	23		23	1.2
313	20	18	20		19	0.7	20	24	22		22	1.2
625	20	17	24		20	2.0	24	26	23		24	0.9
1250	19	19	17		18	0.7	19	20	24		21	1.5
2500	13	10	19	*	14	2.6	18	21	23		21	1.5
5000	M	M	M		-	-	20	17	18	*	18	0.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.307	0.0003	0.27999	0.7716	0.46558
1 - 4	-	4.387	0.0000	-0.05580	0.1767	0.86324
1 - 5	-	4.493	-0.0003	-0.58341	2.5900	0.02243
1 - 3	+	4.735	0.0002	0.30145	0.8365	0.43051
1 - 4	+	4.822	-0.0001	-0.28311	0.9335	0.37258
1 - 5	+	4.811	-0.0001	-0.41705	1.6545	0.12196
1 - 6	+	4.807	-0.0001	-0.66309	3.5434	0.00270

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	15	21	20	19	1.9
9-Aminoacridine	50 µg/pl	-	153	198	161	171	13.9
DMSO	100 µl/pl	+	21	25	23	23	1.2
2-Aminoanthracene	1 µg/pl	+	96	104	112	104	4.6

M = microcolony formation

* = thinning of the background lawn

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[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 5 - Experiment I - Plate incorporation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: WP2 *uvrA* Titre: 257

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	33	26	36		32	3.0	35	36	34	35	0.6
0.00	30	33	35		33	1.5	41	39	37	39	1.2
313	28	32	27		29	1.5	34	32	38	35	1.8
625	28	28	31		29	1.0	32	34	37	34	1.5
1250	22	34	29		28	3.5	30	40	35	35	2.9
2500	25	24	31		27	2.2	37	40	32	36	2.3
5000	27	22	25	*	25	1.5	33	34	39	35	1.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.657	-0.0005	-0.57906	1.8792	0.10229
1 - 4	-	5.597	-0.0003	-0.40047	1.3820	0.19705
1 - 5	-	5.552	-0.0002	-0.46468	1.8921	0.08096
1 - 6	-	5.511	-0.0001	-0.58205	2.8631	0.01127
1 - 3	+	6.188	-0.0006	-0.63576	2.1792	0.06572
1 - 4	+	6.090	-0.0002	-0.35592	1.2044	0.25617
1 - 5	+	6.008	0.0000	-0.08700	0.3149	0.75786
1 - 6	+	5.998	0.0000	-0.08992	0.3611	0.72271

Positive and negative controls Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	33	26	36	32	3.0
MMS	500 µg/pl	-	172	205	179	185	10.0
DMSO	100 µl/pl	+	41	39	37	39	1.2
2-Aminoanthracene	10 µg/pl	+	216	197	199	204	6.0

* = thinning of the background lawn

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 6 - Experiment I - Plate incorporation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA98 Titre: 234

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	34	35	37		35	0.9	40	40	43	41	1.0
0.00	38	31	35		35	2.0	44	48	40	44	2.3
313	33	34	30		32	1.2	42	38	39	40	1.2
625	33	35	29		32	1.8	39	38	41	39	0.9
1250	29	28	34		30	1.9	42	39	44	42	1.5
2500	28	32	31		30	1.2	39	37	40	39	0.9
5000	29	26	30	*	28	1.2	42	45	44	44	0.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.850	-0.0003	-0.35732	1.0122	0.34514
1 - 4	-	5.839	-0.0003	-0.50256	1.8382	0.09588
1 - 5	-	5.777	-0.0001	-0.47401	1.9410	0.07426
1 - 6	-	5.746	-0.0001	-0.59773	2.9824	0.00880
1 - 3	+	6.578	-0.0006	-0.61722	2.0755	0.07659
1 - 4	+	6.460	-0.0001	-0.18022	0.5794	0.57515
1 - 5	+	6.464	-0.0001	-0.39353	1.5434	0.14671
1 - 6	+	6.374	0.0000	0.18269	0.7433	0.46808

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	38	31	35	35	2.0
2-Nitrofluorene	2 µg/pl	-	202	191	194	196	3.3
DMSO	100 µl/pl	+	44	48	40	44	2.3
2-Aminoanthracene	1 µg/pl	+	566	589	561	572	8.6

* = thinning of the background lawn

RTC Report No.: 8837-001-M-06001

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 7 - Experiment I - Plate incorporation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA100 Titre: 223

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	116	134	131		127	5.6	135	140	148	141	3.8
0.00	131	140	136		136	2.6	139	147	138	141	2.8
313	121	111	133		122	6.4	142	155	141	146	4.5
625	127	120	131		126	3.2	157	169	140	155	8.4
1250	150	141	152		148	3.4	131	144	146	140	4.7
2500	127	129	132		129	1.5	140	146	136	141	2.9
5000	76	107	94	*	92	9.0	131	138	130	133	2.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	11.509	-0.0007	-0.45436	1.3495	0.21920
1 - 4	-	11.212	0.0005	0.50043	1.8279	0.09752
1 - 5	-	11.423	0.0001	0.12415	0.4511	0.65932
1 - 6	-	11.731	-0.0003	-0.69321	3.8473	0.00142
1 - 3	+	11.857	0.0009	0.57059	1.8382	0.10863
1 - 4	+	12.087	0.0000	-0.04344	0.1375	0.89337
1 - 5	+	12.110	-0.0001	-0.21445	0.7916	0.44277
1 - 6	+	12.128	-0.0001	-0.49967	2.3074	0.03474

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	116	134	131	127	5.6
Sodium Azide	1 µg/pl	-	938	955	918	937	10.7
DMSO	100 µl/pl	+	139	147	138	141	2.8
2-Aminoanthracene	1 µg/pl	+	1138	1148	1104	1130	13.3

* = thinning of the background lawn

RTC Report No.: 8837-001-M-06001

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 8 - Experiment II - Preincubation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA1535 Titre: 221

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	18	17	19		18	0.6	21	25	18		21	2.0
0.00	15	11	14		13	1.2	20	20	16		19	1.3
313	11	11	8		10	1.0	15	14	15		15	0.3
625	18	16	22		19	1.8	12	17	20		16	2.3
1250	10	10	13		11	1.0	16	20	18		18	1.2
2500	13	14	11		13	0.9	18	16	17	*	17	0.6
5000	12	10	11	*	11	0.6	10	12	10	*	11	0.7

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	3.370	0.0011	0.50640	1.5538	0.16419
1 - 4	-	3.648	-0.0001	-0.07259	0.2301	0.82262
1 - 5	-	3.630	0.0000	-0.07187	0.2598	0.79907
1 - 6	-	3.648	-0.0001	-0.24416	1.0071	0.32887
1 - 3	+	4.202	-0.0005	-0.35413	1.0019	0.34977
1 - 4	+	4.077	0.0000	0.06222	0.1971	0.84767
1 - 5	+	4.088	0.0000	0.05485	0.1981	0.84605
1 - 6	+	4.219	-0.0002	-0.65141	3.4342	0.00341

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	18	17	19	18	0.6
Sodium Azide	1 µg/pl	-	635	588	638	620	16.2
DMSO	50 µl/pl	+	20	20	16	19	1.3
2-Aminoanthracene	1 µg/pl	+	106	94	110	103	4.8

* = thinning of the background lawn

RTC Report No.: 8837-001-M-06001

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 9 - Experiment II - Preincubation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA1537 Titre: 219

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	13	17	16		15	1.2	22	26	28		25	1.8
0.00	21	19	18		19	0.9	23	23	26		24	1.0
156	22	13	16		17	2.6	NT	NT	NT		-	-
313	15	11	17		14	1.8	20	22	25		22	1.5
625	12	15	18		15	1.7	20	23	20		21	1.0
1250	3	6	5	*	5	0.9	22	19	23		21	1.2
2500	3	3	4	*	3	0.3	18	13	10	*	14	2.3
5000	NT	NT	NT		-	-	5	5	2	*	4	1.0

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.400	-0.0020	-0.60633	2.0173	0.08346
1 - 4	-	4.259	-0.0008	-0.47015	1.6845	0.12298
1 - 5	-	4.448	-0.0017	-0.88023	6.6880	0.00002
1 - 6	-	4.217	-0.0011	-0.89230	7.9064	0.00000
1 - 3	+	4.891	-0.0005	-0.59668	1.9673	0.08985
1 - 4	+	4.820	-0.0002	-0.45568	1.6188	0.13655
1 - 5	+	4.928	-0.0005	-0.81716	5.1115	0.00020
1 - 6	+	5.023	-0.0006	-0.95111	12.3186	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	21	19	18	19	0.9
9-Aminoacridine	50 µg/pl	-	142	147	171	153	9.0
DMSO	50 µl/pl	+	23	23	26	24	1.0
2-Aminoanthracene	1 µg/pl	+	98	90	105	98	4.3

NT = not tested
* = thinning of the background lawn

RTC Report No.: 8837-001-M-06001

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 10 - Experiment II - Preincubation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: WP2 *uvrA* Titre: 263

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	33	34	28		32	1.9	30	36	41		36	3.2
0.00	31	31	27		30	1.3	31	38	32		34	2.2
313	31	32	27		30	1.5	31	39	42		37	3.3
625	25	27	23		25	1.2	31	33	34		33	0.9
1250	25	26	26		26	0.3	34	32	33		33	0.6
2500	17	16	28		20	3.8	24	27	24		25	1.0
5000	19	21	23	*	21	1.2	25	18	17	*	20	2.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.528	-0.0007	-0.64475	2.2316	0.06083
1 - 4	-	5.442	-0.0004	-0.62352	2.5221	0.03028
1 - 5	-	5.454	-0.0004	-0.73080	3.8603	0.00197
1 - 6	-	5.302	-0.0002	-0.66465	3.5583	0.00262
1 - 3	+	5.910	-0.0001	-0.10318	0.2745	0.79165
1 - 4	+	5.911	-0.0001	-0.21431	0.6938	0.50359
1 - 5	+	6.013	-0.0004	-0.75510	4.1527	0.00114
1 - 6	+	5.974	-0.0003	-0.87026	7.0667	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	33	34	28	32	1.9
MMS	500 µg/pl	-	218	215	224	219	2.6
DMSO	50 µl/pl	+	31	38	32	34	2.2
2-Aminoanthracene	20 µg/pl	+	191	195	195	194	1.3

* = thinning of the background lawn

RTC Report No.: 8837-001-M-06001

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 11 - Experiment II - Preincubation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA98 Titre: 236

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	37	33	38		36	1.5	41	48	39		43	2.7
0.00	34	39	30		34	2.6	37	37	48		41	3.7
313	30	28	36		31	2.4	40	35	37		37	1.5
625	30	37	30		32	2.3	40	39	43		41	1.2
1250	28	25	30		28	1.5	40	38	36		38	1.2
2500	19	20	21	*	20	0.6	33	36	34		34	0.9
5000	9	4	6	*	6	1.5	13	21	17	*	17	2.3

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.793	-0.0003	-0.22037	0.5977	0.56884
1 - 4	-	5.831	-0.0004	-0.56906	2.1884	0.05348
1 - 5	-	5.879	-0.0005	-0.87408	6.4877	0.00002
1 - 6	-	5.976	-0.0007	-0.96407	14.5157	0.00000
1 - 3	+	6.277	0.0000	0.01527	0.0404	0.96890
1 - 4	+	6.304	-0.0001	-0.16550	0.5307	0.60723
1 - 5	+	6.341	-0.0002	-0.54768	2.3601	0.03457
1 - 6	+	6.533	-0.0004	-0.90061	8.2884	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	34	39	30	34	2.6
2-Nitrofluorene	2 µg/pl	-	193	188	207	196	5.7
DMSO	50 µl/pl	+	37	37	48	41	3.7
2-Aminoanthracene	2 µg/pl	+	670	652	635	652	10.1

* = thinning of the background lawn

[redacted] BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 12 - Experiment II - Preincubation method -

STUDY NO.: 8837-001

SOLVENT: DMSO

Strain: TA100 Titre: 223

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	153	142	139		145	4.3	160	152	163		158	3.3
0.00	142	142	150		145	2.7	167	159	166		164	2.5
313	128	130	126		128	1.2	139	140	139		139	0.3
625	124	125	132		127	2.5	155	140	136		144	5.8
1250	131	134	128		131	1.7	127	134	139		133	3.5
2500	103	92	96	*	97	3.2	118	119	113	*	117	1.9
5000	45	47	48	*	47	0.9	94	98	71	*	88	8.4

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	11.916	-0.0012	-0.83167	3.9627	0.00544
1 - 4	-	11.706	-0.0004	-0.49474	1.8002	0.10201
1 - 5	-	11.878	-0.0007	-0.89317	7.1610	0.00001
1 - 6	-	12.067	-0.0010	-0.97588	17.8805	0.00000
1 - 3	+	12.610	-0.0013	-0.69422	2.5519	0.03800
1 - 4	+	12.492	-0.0008	-0.74892	3.5739	0.00506
1 - 5	+	12.422	-0.0007	-0.88148	6.7303	0.00001
1 - 6	+	12.379	-0.0006	-0.93607	10.6428	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	153	142	139	145	4.3
Sodium Azide	1 µg/pl	-	934	926	920	927	4.1
DMSO	50 µl/pl	+	167	159	166	164	2.5
2-Aminoanthracene	2 µg/pl	+	989	985	992	989	2.0

* = thinning of the background lawn

9. APPENDIX I - Historical Control Data

WITHOUT METABOLIC ACTIVATION

	Untreated *Plate incorporation*	**Untreated** *Pre-incubation*	**Positive control** *Plate incorporation*	**Positive control** *Pre-incubation*
TA1535				
Mean value	19	19	520	516
SD	2.8	2.7	74.5	83.8
n	222	105	222	105
TA1537				
Mean value	17	18	148	123
SD	2.3	1.8	50.3	37.8
n	227	105	227	105
TA98				
Mean value	31	30	224	211
SD	3.1	2.3	31.2	27.0
n	227	102	227	102
TA100				
Mean value	152	135	720	739
SD	18.8	13.7	112.2	128.6
n	228	104	228	104
WP2 *uvr*A				
Mean value	29	30	159	191
SD	5.2	7.0	49.6	111.5
n	6	8	6	8

SD : standard deviation
n : number of experiments

WITH METABOLIC ACTIVATION

	Untreated *Plate incorporation*	Untreated *Pre-incubation*	Positive control *Plate incorporation*	Positive control *Pre-incubation*
TA1535				
Mean value	17	16	154	96
SD	2.4	2.0	32.0	15.5
n	220	105	220	105
TA1537				
Mean value	22	23	120	87
SD	2.8	2.0	25.1	13.6
n	224	103	224	103
TA98				
Mean value	44	42	1079	1008
SD	5.4	4.7	226.2	194.3
n	232	98	232	98
TA100				
Mean value	166	150	1276	1144
SD	18.7	14.9	265.0	181.6
n	235	99	235	99
WP2 *uvrA*				
Mean value	36	37	295	284
SD	8.9	9.4	75.4	91.9
n	6	8	6	8

SD : standard deviation
n : number of experiments

10. APPENDIX II - Study Protocol

RESEARCH TOXICOLOGY CENTRE - ROMA

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

Final protocol
prepared for

AUSIMONT S.p.A.
Via Lombardia, 20
20021 Bollate (MI)
Italy

by

RESEARCH TOXICOLOGY CENTRE S.p.A.
Via Tito Speri, 12
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Italy

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

MANAGEMENT OF STUDY

Scientific Director	:	J. Brightwell, Ph.D.
Head of Genetic and Cellular Toxicology	:	S. Cinelli, Biol.D.
Study Director	:	O. Scarcella, Biol. D.
Sponsor	:	AUSIMONT S.p.A. Via Lombardia, 20 20021 Bollate (MI) Italy
Monitor	:	To be appointed by the Sponsor

QUALITY ASSURANCE

Quality Assurance Manager	:	M. M. Brunetti, Biol.D.

LOCATION OF STUDY

The study will be performed at	:	Research Toxicology Centre S.p.A. Via Tito Speri, 12 00040 Pomezia (Roma) Italy

The laboratory facilities, archives and administration are located at this site.

TIME SCHEDULE OF STUDY

The Study will be conducted with a time schedule agreed between the Sponsor and RTC.

BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

1. INTRODUCTION

1.1 Objective

To assay a number of test items for the ability to induce gene mutations in *Salmonella typhimurium* and *Escherichia coli*, as measured by reversion of auxotrophic strains to prototrophy.

1.2 Regulatory requirements

The study will be conducted in compliance with the GLP regulations of the US FDA [21 CRF part 58, 22 December 1978] and subsequent revisions; with Commission Directive 1999/11/EC of 8 March 1999 (adoption of the "OECD principles on Good Laboratory Practice – as revised in 1997") and subsequent revisions and with Decreto Legislativo 27 Gennaio 1992, no. 120 and subsequent revisions. In addition, the study is designed to comply with the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4D.
- OECD Guidelines for the testing of chemicals No. 471 (Adopted July 1997)

1.3 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2. TEST ITEM

2.1 A number of test items will be supplied for routine testing, each identified by name and relevant univocal identity feature. Documentation of the identity and derivation of each test item will be the responsibility of the Sponsor.

2.2 A study number will be assigned to each test item supplied for investigation. This will consist of a fixed part, identifying the protocol, followed by a sequential number differing for each test item.

2.3 After completion of the study and submission of the final report, all unused samples of each test item will be returned to the Sponsor. An aliquot of each test item will be retained within the archives of the testing facility for a period of ten years after which they will be destroyed.

2.4 Unless otherwise indicated by the Sponsor the storage conditions for the test items will be room temperature.

2.5 The test items will be treated with precautions appropriate for potential carcinogens.

2.6 The amount of each test item received and used will be recorded according to standard procedures.

2.7 Fresh solutions of the test item will be prepared for each day's work; solutions will be prepared on a weight/volume basis without correction for the displacement due to the volume occupied by the test item. Concentrations of solutions will be expressed in terms of active constituents. Preferred solvents will be sterile distilled water, culture medium, DMSO, ethanol, acetone. Other solvents may be used as necessary.

2.8 No assay of test item stability, nor its concentration and homogeneity in vehicle will be undertaken, nor samples of formulated test item consigned to the Sponsor, without express instructions from the Sponsor. No determination of the absorption of the test item in the test system will be made without express instructions from the Sponsor.

3. MATERIALS

3.1 Bacterial strains

Stocks of *Salmonella* tester strains (TA 1535, TA 1537, TA 1538, TA 98, TA 100, TA 97 and TA 102 and some other related strains) were obtained from Dr. B.N.Ames, University of California. Stocks of *E. coli* tester strains (WP2, WP2 *uvr*A and WP2 *uvr*A pKM101) were obtained from Life Science Research, Occold, Suffolk, UK. Permanent stocks are kept at -80°C, and overnight subcultures of these stocks are prepared for each day's work.
The presence of the appropriate genetic markers in these strains is checked on a monthly basis for those in regular use, and as necessary for other strains, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Strain identity is also confirmed by reference to the spontaneous reversion levels and responses to mutagens during use. Bacterial cultures in liquid and on agar are clearly identified with their identity.

Detailed information about the genetic constitution of the tester strains may be found in the cited publications of Dr. B.N.Ames and Drs. M.H.L. Green and W.J. Muriel.

3.2 Media

The following growth media will be used:

Nutrient Broth: Oxoid Nutrient Broth No 2 will be prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This will be used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) will be added to one litre of distilled water and autoclaved.
The solution will then be poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates will be used for the non-selective growth of the tester strains. Incubations on Nutrient Agar will be for approximately 48 or 72 hours.

Minimal Agar: Minimal medium agar will be prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) will be prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. This solution will be autoclaved, and stored. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) will be added to 100 ml of the top agar.

All incubations will be at 37°C.

3.3 S9 mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures. Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor. Records pertaining to the preparation of the S9 fraction are kept in file at RTC. The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	=====
	10.0 ml

3.4 Control substances

Positive control treatments will be used in each experiment. The positive control agents are obtained commercially and characterised by their labelling, and their stability determined from the scientific literature. Sodium azide and methylmethanesulphonate will usually be dissolved in distilled water; 9-aminoacridine, 2-nitrofluorene and 2-aminoanthracene will usually be dissolved in DMSO. The frequency of preparation of stock solutions will be determined by their stability.

4. PRELIMINARY TOXICITY TEST

4.1 Experimental design

In order to establish the concentrations of test item to be used in the main assay, a preliminary toxicity test will be performed.
This test follows the method described in section 6.1, using only one plate per dose level, a single S9 mix concentration (10%) and covering a wide range of concentrations of the test item.

The highest dose-level for this preliminary test, unless limited by the solubility of the test item, will be 5 mg/plate, and the lower dose-levels will be spaced at approximately half-log intervals.

4.2 Selection of dose-levels

The toxicity will be assessed on the basis of a decline in the number of spontaneous revertants or a thinning of the background lawn. The highest dose-level for the mutation assays will be selected as a concentration which elicits moderate toxicity. If there is no evidence of toxicity following treatment with the test item, then the highest dose-level will be 5 mg/plate.

5. EXPERIMENTAL DESIGN

Each experiment will include negative and positive controls, and at least five doses of the test item, tested in the absence and presence of an S9 metabolising system. Three replicate plates will be used at each test point, and two independent experiments will be performed. If a positive result is obtained in any tester strain, a confirmatory experiment will be performed under the same experimental conditions. If, however, negative results are obtained in the first experiment, the confirmatory experiment will be performed using the pre-incubation method. A further experiment may be undertaken if inconsistent results are obtained.

The five bacterial strains *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvr*A will be used in this study.

Negative controls: untreated and solvent vehicle controls will be prepared for each experiment; when the solvent is distilled water, these will be considered to be equivalent and only one set of controls is performed.

Positive controls: treatments are indicated in the following table:

Tester strain	*Absence of S9*	*Presence of S9*
TA1535	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate
TA100	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate (2 µg/plate)
TA1537	9-amino-acridine 50 µg/plate	2-aminoanthracene 1 µg/plate
TA98	2-nitrofluorene 2 µg/plate	2- aminoanthracene 1 µg/plate (2 µg/plate)
WP2 *uvr*A	methylmethanesulphonate 500 µg/plate	2-aminoanthracene 10 µg/plate (20 µg/plate)

Concentrations refer to both treatment methods. When two values are given, the figures in brackets refer to the pre-incubation method assay.

Test item: the highest dose-level of the test item to be used will be selected as described above. Further dose levels will be selected at intervals of a factor of two.

Where it seems advisable, further test points or controls may be included in experiments.

In addition, plates will be prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures will be plated on nutrient agar plates to establish the number of bacteria in the cultures.

6. ASSAY PROCEDURE

6.1 Plate-incorporation

The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) will be added to molten overlay agar and vortexed. The mixture will then be poured on the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture will be composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control substance solution	0.1 ml
(iii)	S9 mix or phosphate buffer	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The volume of test item solution, as indicated, will usually be 0.1 ml; in the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

6.2 Pre-incubation

The components will be added in turn to an empty test-tube:

(i) Bacterial suspension	0.1 ml
(ii) Test or control substance solution	0.05 ml
(iii) S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The volume of test item solution, as indicated, will usually be 0.05 ml. Where control or test items are dissolved in aqueous solvents, the volume used may be 0.1 ml. In the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

The incubate will be vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar will then be added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

6.3 Incubation and scoring

The prepared plates will be inverted and incubated for approximately 72 hours at 37°C. When the test item is a liquid at ambient temperature, the plates will be incubated in separate closed containers for each dose-level. After this period of incubation, the plates may be held at 4°C prior to scoring. Scoring is effected by counting the number of revertant colonies on each plate, either manually, or using a Cardinal - Automatic colony counting system (Perceptive Instruments). Contaminated plates will be considered on a case-by-case basis.

7. REPORTING

7.1 Presentation of data

The data will be presented in tabular form. The individual plate counts for each experiment will be given, together with the means and standard errors of the means, and regression analyses.

7.2 Evaluation of data

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

Evaluation of Ames test data based on a 'doubling rate' has been shown to be as effective as statistical techniques in allowing the correct interpretation of test results (Chu et al. 1981).

7.3 Historical Data

In any case of unexpected results or analytical findings in treated or untreated plates historical data shall be included for comparison and interpretation.

7.4 Reporting procedure

A draft report will be despatched for comment before finalisation.

7.5 Final report

The following information and data will be included in the final report:

- name and address of the facility performing the study and the dates on which the study was initiated and completed;
- objective and procedures stated in the approved protocol, including approved changes to the original protocol;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test item identified by name;
- method used;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- the name and signature of the Study Director;
- a summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis;
- the location where all raw data, specimens and final report are to be stored;
- Quality Assurance statement.

Three copies of the final report (2 bound, 1 unbound) will be supplied.

7.6 Records kept

Full records will be maintained of all aspects of study conduct, along with the results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the study.

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7.7 Archiving

All raw data, records and documentation arising from this study and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of five years from the date of consignment of the Final Report. At the end of this period, the Sponsor will be contacted for despatch or disposal of the material.

8. STUDY CONDUCT

8.1 Language

English language and Italian language versions of the study protocol, Standard Operating Procedures and other study documents may be used interchangeably. Similarly, English and Italian renderings of chemical names, including that of the test material will be considered to be equivalent.

8.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test items. When the study director considers it advisable to modify the procedures described for the selection of a solvent, selection of dose-levels, interpretation of the outcome of the study or other aspects of the study conduct, he will record carefully the decision he has reached and the reasoning which led to it.
Each scientific decision has to be discussed with the Sponsor before application.

8.3 Quality assurance

The study is subjected to the procedure for quality assurance as defined by the relevant GLP regulations. Specifically:

- the protocol is inspected for compliance;
- procedures of the laboratories concerned will be inspected at intervals adequate to assure the integrity of the study;
- the final report is reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in agreement with the raw data;
- periodic reports on these activities are made to management and the Study Director.

All raw data pertaining to the study will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

9. REFERENCES

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U.K.E.M.S., Swansea, 1983.

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PROTOCOL APPROVAL PAGE

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