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ABM-FD (EXTRACT OF HIMEMATSUTAKE)

BACTERIAL REVERSE MUTATION TEST

Report

**ABM-FD (EXTRACT OF HIMEMATSUTAKE)
BACTERIAL REVERSE MUTATION TEST**

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

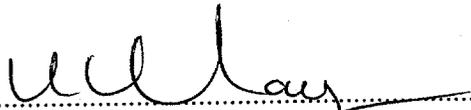
The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

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

EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.



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

.....
11 October 2005

Date

QUALITY ASSURANCE STATEMENT

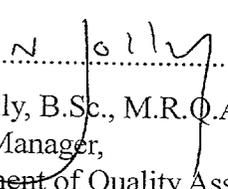
The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol Audit	14 December 2004	14 December 2004
Report Audit	3 – 11 May 2005	11 May 2005

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

Process Based Inspections	Date(s) of Inspection	Date of Reporting to Management
Formulation and Treatment	7 October 2004	7 October 2004
Plate Scoring	17 January 2005	17 January 2005
Clinical Chemistry	10 January 2005	10 January 2005

In addition, an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were promptly reported to Company Management.



 Neal Jolly, B.Sc., M.R.Q.A.,
 Group Manager,
 Department of Quality Assurance,
 Huntingdon Life Sciences Ltd.

..... 11 OCTOBER 2005
 Date

RESPONSIBLE PERSONNEL

STUDY MANAGEMENT

Kenneth May, B.Sc., C.Biol., M.I.Biol.
Study Director

SUMMARY

In this *in vitro* assessment of the mutagenic potential of ABM-FD (Extract of Himematsutake), histidine-dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 *uvrA* (pKM101), were exposed to ABM-FD (Extract of Himematsutake) diluted in water. Water was also used as a negative control.

Following a preliminary toxicity test, two independent mutation tests were performed in the presence and absence of liver preparations (S9 mix) from rats treated with Aroclor 1254. The first test was a standard plate incorporation assay; the second included a pre-incubation stage.

Concentrations of ABM-FD (Extract of Himematsutake) up to 5000 µg/plate were tested. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. Other concentrations used were a series of *ca* half-log₁₀ 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 ABM-FD (Extract of Himematsutake) 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 ABM-FD (Extract of Himematsutake) showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

INTRODUCTION

This report describes a study designed to assess ABM-FD (Extract of Himematsutake) for its ability to cause point (gene) mutation in *Salmonella typhimurium* and *Escherichia coli*. The study was conducted in compliance with the following guidelines:

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

EC Commission Directive 2000/32/EC Annex 4D-B.13/14. Mutagenicity - Reverse mutation test in bacteria. No. L 136/57.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.

Japanese Ministry of Agriculture, Forestry and Fisheries. Test Data for Registration of Agricultural Chemicals, 12 Nohsan No. 8147, Agricultural Production Bureau, November 24, 2000.

Official notice of J MHLW, METI and ME (21 November 2003):

YAKUSHOKUHATSU No.1121002

SEIKYOKU No.2

KANPOKIHATSU No. 031121002

ICH (1996) Guideline S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. PAB/PCD Notification No. 444.

ICH (1998) Guideline S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. PMSB/ELD Notification No. 554.

The procedures also met the requirements of the FDA (United States Food and Drug Administration) "Redbook 2000" Toxicological Principles for the Safety Assessment of Food Ingredients.

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 and so forms part of the general requirements for registration.

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* that are incapable of synthesising the amino acid, tryptophan, which is required for growth.

The strains used carry additional mutations that 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.

Some 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 homogenate fraction (S9 mix).

The protocol was approved by Huntingdon Life Sciences Management on 25 November 2004, by the Sponsor on 8 December 2004 and by the Study Director on 9 December 2004.

The study was conducted at Huntingdon Life Sciences Ltd., Eye Research Centre, Eye, Suffolk, IP23 7PX, England, except for formulation chemistry which was conducted at Huntingdon Life Sciences Ltd., Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE28 4HS.

Experimental start date: 14 December 2004.

Experimental completion date: 3 March 2005.

TEST SUBSTANCE

Identity: ABM-FD (Extract of Himematsutake)

Appearance: Brown solid

Storage conditions: Room temperature, in the dark

Batch number: EX200408

Expiry date: End of July 2006

Purity: 2.9 g / 100 g as beta-glucan

Date received: 31 August 2004

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The genotype of each bacterial strain used in this study is outlined in the following table:

Species	Strain	Genotype	
<i>Salmonella typhimurium</i>	TA1535	<i>hisG46</i>	<i>rfa</i> Δ <i>uvrB</i>
<i>Salmonella typhimurium</i>	TA1537	<i>hisC3076</i>	<i>rfa</i> Δ <i>uvrB</i>
<i>Salmonella typhimurium</i>	TA98	<i>hisD3052</i>	<i>rfa</i> Δ <i>uvrB</i> (pKM101)
<i>Salmonella typhimurium</i>	TA100	<i>hisG46</i>	<i>rfa</i> Δ <i>uvrB</i> (pKM101)
<i>Escherichia coli</i>	WP2 <i>uvrA</i> (pKM101)	<i>trpE</i>	Ochre <i>uvrA</i> (pKM101)

Note: The deletion (Δ) through *uvrB* also affects the nitrate reductase (*chlA*) and biotin (*bio*) genes

These strains were used to detect base changes and frameshift mutations as follows:

base change mutagens: *S. typhimurium* TA1535 and TA100, and *E. coli* WP2 *uvrA* (pKM101).

frameshift mutagens: *S. typhimurium* TA1537, TA98 and TA100.

The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England.

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 stored as aliquots of nutrient broth cultures at *ca* -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 were also assessed.

For use in tests, an aliquot of frozen culture was added to 25 mL of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures were intended to provide approximately 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 and counting the resultant colonies.

POSITIVE CONTROLS**In the absence of S9 mix**

Identity: Sodium azide
CAS No.: 26628-22-8
Supplier: Sigma-Aldrich Chemical Company
Lot number: 77H0079
Purity: 99.5%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 2 µg/plate for strains TA100 and TA1535

Identity: 9-Aminoacridine
CAS No.: 90-45-9
Supplier: Sigma-Aldrich Chemical Company
Lot number: 106F06682
Purity: > 97%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 50 µg/plate for strain TA1537

Identity: 2-Nitrofluorene
CAS No.: 607-57-8
Supplier: Sigma-Aldrich Chemical Company
Lot number: S08447-022
Purity: 98%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 2 µg/plate for strain TA98

Identity: 4-Nitroquinoline-1-oxide
CAS No.: 56-57-5
Supplier: Sigma-Aldrich Chemical Company
Lot number: 102K0996
Purity: 99%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 2 µg/plate for strain WP2 *uvrA* (pKM101)

In the presence of S9 mix

Identity: 2-Aminoanthracene
CAS No.: 613-13-8
Supplier: Sigma-Aldrich Chemical Company
Lot number: S11804-252
Purity: 96%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 5 µg/plate for strains TA100 and TA1535
10 µg/plate for strain WP2 *uvrA* (pKM101)

Identity: Benzo[a]pyrene
CAS No.: 50-32-8
Supplier: Sigma-Aldrich Chemical Company
Lot number: 17776-111
Purity: 97%
Solvent: DMSO (Sigma-Aldrich, A.C.S. spectrophotometric grade)
Concentration: 5 µg/plate for strains TA98 and TA1537

PREPARATION OF S9 FRACTION

S9 fraction, prepared from male Sprague-Dawley derived rats, dosed with Aroclor to stimulate mixed-function oxidases in the liver, was purchased from Molecular Toxicology Incorporated, USA and stored at *ca* -80°C. The quality control statements relating to the batches of S9 preparation used are included in the raw data and copies are included in this report as Appendix 3.

Lot No.: 1797 (Date of preparation: 2 December 2004)

Lot No.: 1760 (Date of preparation: 9 September 2004)

Lot No.: 1817 (Date of preparation: 25 January 2005)

PREPARATION OF S9 MIX

The S9 mix contained: S9 fraction (10% v/v), MgCl₂ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM) in water. All the cofactors were sterilised before use.

SELECTION OF VEHICLE AND FORMULATION OF TEST SUBSTANCE

Water (purified in-house by reverse osmosis) was used as the vehicle for ABM-FD (Extract of Himematsutake) in this study.

The highest concentration of ABM-FD (Extract of Himematsutake) tested in this study was 50 mg/mL in the chosen vehicle, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. The highest concentration in each test was diluted with water to produce a series of lower concentrations, separated by *ca* half-log₁₀ intervals.

All concentrations cited in this report are expressed in terms of the ABM-FD (Extract of Himematsutake) sample as received.

MUTATION TEST PROCEDURE

Preliminary toxicity test

Aliquots of 0.1 mL of the test substance solutions (seven concentrations up to 5000 µg/plate), positive control or negative control were placed in glass vessels. The negative control was the chosen vehicle, water. S9 mix (0.5 mL) or 0.1 M pH 7.4 phosphate buffer (0.5 mL) was added, followed by 0.1 mL of a 10 hour bacterial culture and 2 mL of agar containing histidine

(0.05 mM), biotin (0.05 mM) and tryptophan (0.05 mM). 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 contents of the dish. A single Petri dish was 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 sodium phosphate buffer. All plates were incubated at approximately 37°C for *ca* 72 hours. After this period, the appearance of the background bacterial lawn was examined and revertant colonies counted either using an automated colony counter (Perceptive Instruments *Domino*) or manually.

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 maximum concentration selected for use in the main mutation tests would be the same as that used in the first. If toxic effects were observed, a lower concentration might be chosen, ensuring that signs of bacterial inhibition were present at this maximum concentration. Ideally, a minimum of four non-toxic concentrations should be obtained. If precipitate were observed on the plates at the end of the incubation period, at least four non-precipitating concentrations should be obtained, unless otherwise justified by the Study Director.

First main mutation test

The first main mutation test was conducted using the same procedures as the preliminary test, except that only five concentrations from 50 to 5000 µg/plate were used, and three Petri dishes were prepared for each treatment.

Second main mutation test

As a clear negative response was obtained in the first test, a variation to the test procedure was used for the second test. The variation used was the pre-incubation assay in which the tubes, which contained mixtures of bacteria, buffer or S9 mix and test dilution, were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay. The maximum concentration chosen was again 5000 µg/plate, and only five concentrations were used.

STABILITY, HOMOGENEITY AND FORMULATION ANALYSIS

The stability of ABM-FD (Extract of Himematsutake) was not determined as part of this study. The stability and homogeneity of ABM-FD (Extract of Himematsutake) in the vehicle were determined as part of Huntingdon Life Sciences Study No. IKI/001. Analysis of the achieved concentration of test formulations was performed as part of this study. Results of this analysis are presented in Appendix 4. All results are considered acceptable, within the limitations of the assay.

ASSESSMENT OF RESULTS

Acceptance criteria

For a test to be considered valid, the mean of the vehicle control revertant colony numbers for each strain should lie within or close to the 99% confidence limits of the current historical control range of the laboratory unless otherwise justified by the Study Director. The historical range is maintained as a rolling record over a maximum of five years. Also, the positive control compounds must induce an increase in mean revertant colony numbers of at least twice (three times in the case of strains TA1535 and TA1537) the concurrent vehicle controls. Viable cell counts in the 10-hour bacterial cultures must be at least 10^9 /mL.

Analysis of data

The mean number and standard deviation of revertant colonies were calculated for all groups. The means for all treatment groups were compared with those obtained for the vehicle control groups.

Criteria for assessing mutagenic potential

If exposure to a test substance produces a reproducible increase in revertant colony numbers of at least twice (three times in the case of strains TA1535 and TA1537) the concurrent vehicle controls, with some evidence of a positive dose-response relationship, it is considered to exhibit mutagenic activity in this test system. No statistical analysis is performed.

If exposure to a test substance does not produce a reproducible increase in revertant colony numbers, it is considered to show no evidence of mutagenic activity in this test system. No statistical analysis is performed.

If the results obtained had failed to satisfy the criteria for a clear "positive" or "negative" response, even after additional testing, the test data may have been subjected to analysis to determine the statistical significance of any increases in revertant colony numbers. The statistical procedures used would have been those described by Mahon *et al* (1989) and are usually Dunnett's test followed, if appropriate, by trend analysis. Biological importance should always be considered along with statistical significance. In general, treatment-associated increases in revertant colony numbers below two or three times the vehicle controls (as described above) are not considered biologically important. It should be noted that it would have been acceptable to conclude an equivocal response if no clear results could be obtained.

Occasionally, these criteria may not be appropriate to the test data; if this had been the case, the Study Director would have used his/her scientific judgement.

DEVIATIONS FROM PROTOCOL

There were no deviations from the protocol.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor. Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and 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 will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

The results obtained with ABM-FD (Extract of Himematsutake) and positive control compounds are presented in Tables 1 to 15. 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 of the S9 mix, buffer and test substance formulation.

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 vehicle controls were within or close to the 99% confidence limits of the current historical control range of the laboratory (Appendix 1). 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.

PRELIMINARY TOXICITY TEST (Tables 1 to 5)

No evidence of toxicity was obtained following exposure to ABM-FD (Extract of Himematsutake). A maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the main mutation tests.

FIRST MAIN MUTATION TEST (Tables 6 to 10)

No evidence of toxicity was obtained following exposure to ABM-FD (Extract of Himematsutake).

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to ABM-FD (Extract of Himematsutake) at any concentration up to 5000 µg/plate in either the presence or absence of S9 mix.

SECOND MAIN MUTATION TEST (Tables 11 to 15)

No evidence of toxicity was obtained following exposure to ABM-FD (Extract of Himematsutake).

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to ABM-FD (Extract of Himematsutake) at any concentration up to 5000 µg/plate in either the presence or absence of S9 mix.

CONCLUSION

It is concluded that ABM-FD (Extract of Himematsutake) showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

REFERENCES

AMES, B.N., McCANN, J. and YAMASAKI, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutation Res.* **31**, 347-364.

GREEN, M.H.L. (1984) Mutagen testing using *trp*⁺ reversion in *Escherichia coli* in KILBEY, B.J., LEGATOR, M., NICHOLS, W. and RAMEL, C. (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, pp.161-187. Elsevier Science Publishers BV, Amsterdam.

MAHON, G.A.T., GREEN, M.H.L., MIDDLETON, B., MITCHELL, I. de G., ROBINSON, W.D. and TWEATS, D.J. (1989) Analysis of data from microbial colony assays in: KIRKLAND, D.J. (Ed.). *UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Report. Part III. Statistical Evaluation of Mutagenicity Test Data*, pp.26-65. Cambridge University Press, Cambridge.

MARON, D.M. and AMES, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.* **113**, 173-215.

TABLE 1: Results obtained with *S. typhimurium* TA98: preliminary toxicity test

Tester strain: <i>S. typhimurium</i> TA98				
Test substance: ABM-FD (Extract of Himematsutake)				
Test No.: Preliminary				
Addition	S9 mix + present - absent	Revertant colony counts/plate	Fold increase relative to vehicle	
		Individual plate counts		
Water (0.1 mL/plate)	-	26	-	
Test substance (5 µg/plate)	-	39	1.5	
Test substance (15 µg/plate)	-	28	1.1	
Test substance (50 µg/plate)	-	30	1.2	
Test substance (150 µg/plate)	-	41	1.6	
Test substance (500 µg/plate)	-	45	1.7	
Test substance (1500 µg/plate)	-	30	1.2	
Test substance (5000 µg/plate)	-	45	1.7	
Water (0.1 mL/plate)	+	59	-	
Test substance (5 µg/plate)	+	44	0.7	
Test substance (15 µg/plate)	+	37	0.6	
Test substance (50 µg/plate)	+	52	0.9	
Test substance (150 µg/plate)	+	57	1.0	
Test substance (500 µg/plate)	+	49	0.8	
Test substance (1500 µg/plate)	+	46	0.8	
Test substance (5000 µg/plate)	+	66	1.1	
2-Nitrofluorene (2 µg/plate)	-	370	14.2	
Benzo[a]pyrene (5 µg/plate)	+	811	13.7	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	139	-
Sterility checks (total colony counts):				
Buffer	-	0	-	
S9 mix	+	0	-	
Test substance (5000 µg/plate)	-	0	-	

TABLE 2: Results obtained with *S. typhimurium* TA100: preliminary toxicity test

Tester strain: <i>S. typhimurium</i> TA100			
Test substance: ABM-FD (Extract of Himematsutake)			
Test No.: Preliminary			
Addition	S9 mix + present - absent	Revertant colony counts/plate	Fold increase relative to vehicle
		Individual plate counts	
Water (0.1 mL/plate)	-	143	-
Test substance (5 µg/plate)	-	129	0.9
Test substance (15 µg/plate)	-	166	1.2
Test substance (50 µg/plate)	-	140	1.0
Test substance (150 µg/plate)	-	153	1.1
Test substance (500 µg/plate)	-	161	1.1
Test substance (1500 µg/plate)	-	161	1.1
Test substance (5000 µg/plate)	-	170	1.2
Water (0.1 mL/plate)	+	199	-
Test substance (5 µg/plate)	+	193	1.0
Test substance (15 µg/plate)	+	181	0.9
Test substance (50 µg/plate)	+	195	1.0
Test substance (150 µg/plate)	+	197	1.0
Test substance (500 µg/plate)	+	187	0.9
Test substance (1500 µg/plate)	+	216	1.1
Test substance (5000 µg/plate)	+	224	1.1
Sodium azide (2 µg/plate)	-	748	5.2
2-Aminoanthracene (5 µg/plate)	+	3034	15.2
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		253	-
Sterility checks (total colony counts):			
Buffer	-	0	-
S9 mix	+	0	-
Test substance (5000 µg/plate)	-	0	-

TABLE 3: Results obtained with *S. typhimurium* TA1535: preliminary toxicity test

Tester strain: <i>S. typhimurium</i> TA1535				
Test substance: ABM-FD (Extract of Himematsutake)				
Test No.: Preliminary				
Addition		S9 mix	Revertant colony counts/plate	Fold increase relative to vehicle
		+ present - absent	Individual plate counts	
Water	(0.1 mL/plate)	-	15	-
Test substance	(5 µg/plate)	-	14	0.9
Test substance	(15 µg/plate)	-	14	0.9
Test substance	(50 µg/plate)	-	21	1.4
Test substance	(150 µg/plate)	-	22	1.5
Test substance	(500 µg/plate)	-	15	1.0
Test substance	(1500 µg/plate)	-	9	0.6
Test substance	(5000 µg/plate)	-	21	1.4
Water	(0.1 mL/plate)	+	16	-
Test substance	(5 µg/plate)	+	19	1.2
Test substance	(15 µg/plate)	+	22	1.4
Test substance	(50 µg/plate)	+	17	1.1
Test substance	(150 µg/plate)	+	21	1.3
Test substance	(500 µg/plate)	+	23	1.4
Test substance	(1500 µg/plate)	+	21	1.3
Test substance	(5000 µg/plate)	+	21	1.3
Sodium azide	(2 µg/plate)	-	1322	88.1
2-Aminoanthracene	(5 µg/plate)	+	292	18.3
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	208	-
Sterility checks (total colony counts):				
Buffer		-	0	-
S9 mix		+	0	-
Test substance	(5000 µg/plate)	-	0	-

TABLE 4: Results obtained with *S. typhimurium* TA1537: preliminary toxicity test

Tester strain: <i>S. typhimurium</i> TA1537				
Test substance: ABM-FD (Extract of Himematsutake)				
Test No.: Preliminary				
Addition		S9 mix + present - absent	Revertant colony counts/plate	Fold increase relative to vehicle
			Individual plate counts	
Water	(0.1 mL/plate)	-	19	-
Test substance	(5 µg/plate)	-	15	0.8
Test substance	(15 µg/plate)	-	15	0.8
Test substance	(50 µg/plate)	-	15	0.8
Test substance	(150 µg/plate)	-	13	0.7
Test substance	(500 µg/plate)	-	12	0.6
Test substance	(1500 µg/plate)	-	19	1.0
Test substance	(5000 µg/plate)	-	12	0.6
Water	(0.1 mL/plate)	+	41	-
Test substance	(5 µg/plate)	+	36	0.9
Test substance	(15 µg/plate)	+	43	1.0
Test substance	(50 µg/plate)	+	42	1.0
Test substance	(150 µg/plate)	+	28	0.7
Test substance	(500 µg/plate)	+	37	0.9
Test substance	(1500 µg/plate)	+	38	0.9
Test substance	(5000 µg/plate)	+	43	1.0
9-Aminoacridine	(50 µg/plate)	-	637	33.5
Benzo[a]pyrene	(5 µg/plate)	+	166	4.0
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	168	-
Sterility checks (total colony counts):				
Buffer		-	0	-
S9 mix		+	0	-
Test substance	(5000 µg/plate)	-	0	-

TABLE 5: Results obtained with *E. coli* WP2 *uvrA* (pKM101): preliminary toxicity test

Tester strain: <i>E. coli</i> WP2 <i>uvrA</i> (pKM101)				
Test substance: ABM-FD (Extract of Himematsutake)				
Test No.: Preliminary				
Addition	S9 mix + present - absent	Revertant colony counts/plate		Fold increase relative to vehicle
		Individual plate counts		
Water	(0.1 mL/plate)	-	119	-
Test substance	(5 µg/plate)	-	77	0.6
Test substance	(15 µg/plate)	-	85	0.7
Test substance	(50 µg/plate)	-	88	0.7
Test substance	(150 µg/plate)	-	108	0.9
Test substance	(500 µg/plate)	-	100	0.8
Test substance	(1500 µg/plate)	-	114	1.0
Test substance	(5000 µg/plate)	-	75	0.6
Water	(0.1 mL/plate)	+	146	-
Test substance	(5 µg/plate)	+	104	0.7
Test substance	(15 µg/plate)	+	124	0.8
Test substance	(50 µg/plate)	+	126	0.9
Test substance	(150 µg/plate)	+	150	1.0
Test substance	(500 µg/plate)	+	130	0.9
Test substance	(1500 µg/plate)	+	144	1.0
Test substance	(5000 µg/plate)	+	150	1.0
4-Nitroquinoline-1-oxide	(2 µg/plate)	-	2012	16.9
2-Aminoanthracene	(10 µg/plate)	+	321	2.2
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	215	-
Sterility checks (total colony counts):				
Buffer		-	0	-
S9 mix		+	0	-
Test substance	(5000 µg/plate)	-	0	-

TABLE 6: Results obtained with *S. typhimurium* TA98: main mutation test 1

Tester strain: <i>S. typhimurium</i> TA98									
Test substance: ABM-FD (Extract of Himematsutake)									
Test No.: Main test 1									
Addition	S9 mix + present - absent	Revertant colony counts/plate					Mean	Standard Deviation	Fold increase relative to vehicle
		Individual plate counts							
Water (0.1 mL/plate)	-	53	43	49	48	5	-		
Test substance (50 µg/plate)	-	46	75	60	60	15	1.3		
Test substance (150 µg/plate)	-	60	57	60	59	2	1.2		
Test substance (500 µg/plate)	-	49	60	65	58	8	1.2		
Test substance (1500 µg/plate)	-	10	24	24	19	8	0.4		
Test substance (5000 µg/plate)	-	36	30	28	31	4	0.6		
Water (0.1 mL/plate)	+	68	72	73	71	3	-		
Test substance (50 µg/plate)	+	32	48	58	46	13	0.6		
Test substance (150 µg/plate)	+	75	55	57	62	11	0.9		
Test substance (500 µg/plate)	+	65	75	65	68	6	1.0		
Test substance (1500 µg/plate)	+	85	77	81	81	4	1.1		
Test substance (5000 µg/plate)	+	89	107	85	94	12	1.3		
2-Nitrofluorene (2 µg/plate)	-	378	366	528	424	90	8.8		
Benzo[a]pyrene (5 µg/plate)	+	226	234	187	216	25	3.0		
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	152	152	144	149	5	-	
Sterility checks (total colony counts):									
Buffer	-	0	0	0	0	0	-		
S9 mix	+	0	0	0	0	0	-		
Test substance (5000 µg/plate)	-	0	0	0	0	0	-		

TABLE 7: Results obtained with *S. typhimurium* TA100: main mutation test 1

Tester strain: <i>S. typhimurium</i> TA100								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 1								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	157	162	157	159	3	-	
Test substance (50 µg/plate)	-	182	170	186	179	8	1.1	
Test substance (150 µg/plate)	-	158	188	162	169	16	1.1	
Test substance (500 µg/plate)	-	174	165	172	170	5	1.1	
Test substance (1500 µg/plate)	-	143	173	166	161	16	1.0	
Test substance (5000 µg/plate)	-	181	162	152	165	15	1.0	
Water (0.1 mL/plate)	+	201	190	198	196	6	-	
Test substance (50 µg/plate)	+	135	148	147	143	7	0.7	
Test substance (150 µg/plate)	+	158	174	148	160	13	0.8	
Test substance (500 µg/plate)	+	164	160	147	157	9	0.8	
Test substance (1500 µg/plate)	+	183	176	175	178	4	0.9	
Test substance (5000 µg/plate)	+	227	213	182	207	23	1.1	
Sodium azide (2 µg/plate)	-	586	771	816	724	122	4.6	
2-Aminoanthracene (5 µg/plate)	+	1416	1547	1563	1509	81	7.7	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	145	170	165	160	13	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

TABLE 8: Results obtained with *S. typhimurium* TA1535: main mutation test 1

Tester strain: <i>S. typhimurium</i> TA1535								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 1								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	21	35	29	28	7	-	
Test substance (50 µg/plate)	-	22	27	23	24	3	0.9	
Test substance (150 µg/plate)	-	22	27	26	25	3	0.9	
Test substance (500 µg/plate)	-	23	21	13	19	5	0.7	
Test substance (1500 µg/plate)	-	16	14	29	20	8	0.7	
Test substance (5000 µg/plate)	-	20	27	35	27	8	1.0	
Water (0.1 mL/plate)	+	30	26	23	26	4	-	
Test substance (50 µg/plate)	+	14	20	10	15	5	0.6	
Test substance (150 µg/plate)	+	20	14	8	14	6	0.5	
Test substance (500 µg/plate)	+	17	19	22	19	3	0.7	
Test substance (1500 µg/plate)	+	19	20	15	18	3	0.7	
Test substance (5000 µg/plate)	+	31	28	31	30	2	1.2	
Sodium azide (2 µg/plate)	-	1088	942	868	966	112	34.5	
2-Aminoanthracene (5 µg/plate)	+	290	281	366	312	47	12.0	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	225	213	186	208	20	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

TABLE 9: Results obtained with *S. typhimurium* TA1537: main mutation test 1

Tester strain: <i>S. typhimurium</i> TA1537												
Test substance: ABM-FD (Extract of Himematsutake)												
Test No.: Main test 1												
Addition	S9 mix + present - absent	Revertant colony counts/plate					Mean	Standard Deviation	Fold increase relative to vehicle			
		Individual plate counts										
Water	(0.1 mL/plate)	-	19	24	22	22	3	-				
Test substance	(50 µg/plate)	-	29	16	13	19	9	0.9				
Test substance	(150 µg/plate)	-	16	23	12	17	6	0.8				
Test substance	(500 µg/plate)	-	16	10	19	15	5	0.7				
Test substance	(1500 µg/plate)	-	21	19	15	18	3	0.8				
Test substance	(5000 µg/plate)	-	31	22	34	29	6	1.3				
Water	(0.1 mL/plate)	+	39	42	39	40	2	-				
Test substance	(50 µg/plate)	+	48	32	34	38	9	1.0				
Test substance	(150 µg/plate)	+	32	37	37	35	3	0.9				
Test substance	(500 µg/plate)	+	46	38	31	38	8	1.0				
Test substance	(1500 µg/plate)	+	41	30	32	34	6	0.9				
Test substance	(5000 µg/plate)	+	46	38	42	42	4	1.1				
9-Aminoacridine	(50 µg/plate)	-	525	254	211	330	170	15.0				
Benzo[a]pyrene	(5 µg/plate)	+	219	222	209	217	7	5.4				
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)			-	245	a	241	a	253	a	246	6	-
				140	b	187	b	201	b	176	32	-
Sterility checks (total colony counts):												
Buffer		-	0	0	0	0	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	0	0	0	0	-

a Culture used for testing in the absence of S9 mix

b Culture used for testing in the presence of S9 mix (tested on a separate occasion, since the original test was declared invalid because of inadequate positive control response)

TABLE 10: Results obtained with *E. coli* WP2 *uvrA* (pKM101): main mutation test 1

Results obtained with <i>E. coli</i> WP2 <i>uvrA</i> (pKM101)									
Test substance: ABM-FD (Extract of Himematsutake)									
Test No.: Main test 1									
Addition	S9 mix + present - absent	Revertant colony counts/plate					Mean	Standard Deviation	Fold increase relative to vehicle
		Individual plate counts							
Water (0.1 mL/plate)	-	121	121	132	125	6	-		
Test substance (50 µg/plate)	-	131	143	115	130	14	1.0		
Test substance (150 µg/plate)	-	130	135	131	132	3	1.1		
Test substance (500 µg/plate)	-	140	112	139	130	16	1.0		
Test substance (1500 µg/plate)	-	88	92	106	95	9	0.8		
Test substance (5000 µg/plate)	-	121	175	125	140	30	1.1		
Water (0.1 mL/plate)	+	206	233	222	220	14	-		
Test substance (50 µg/plate)	+	242	188	206	212	27	1.0		
Test substance (150 µg/plate)	+	290	226	233	250	35	1.1		
Test substance (500 µg/plate)	+	177	205	213	198	19	0.9		
Test substance (1500 µg/plate)	+	177	198	248	208	36	0.9		
Test substance (5000 µg/plate)	+	175	184	197	185	11	0.8		
4-Nitroquinoline-1-oxide (2 µg/plate)	-	1306	1148	965	1140	171	9.1		
2-Aminoanthracene (10 µg/plate)	+	549	545	553	549	4	2.5		
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	302 ^a	320 ^a	293 ^a	305	14	-	
			230 ^b	205 ^b	218 ^b	218	13	-	
Sterility checks (total colony counts):									
Buffer	-	0	0	0	0	0	-		
S9 mix	+	0	0	0	0	0	-		
Test substance (5000 µg/plate)	-	0	0	0	0	0	-		

^a Culture used for testing in the absence of S9 mix

^b Culture used for testing in the presence of S9 mix (tested on a separate occasion, since the original test was declared invalid because of inadequate positive control response)

TABLE 11: Results obtained with *S. typhimurium* TA98: main mutation test 2

Tester strain: <i>S. typhimurium</i> TA98								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 2 (with pre-incubation)								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water	(0.1 mL/plate)	-	45	49	44	46	3	-
Test substance	(50 µg/plate)	-	38	48	37	41	6	0.9
Test substance	(150 µg/plate)	-	42	36	36	38	3	0.8
Test substance	(500 µg/plate)	-	41	37	48	42	6	0.9
Test substance	(1500 µg/plate)	-	36	44	41	40	4	0.9
Test substance	(5000 µg/plate)	-	32	29	37	33	4	0.7
Water	(0.1 mL/plate)	+	60	56	58	58	2	-
Test substance	(50 µg/plate)	+	59	52	57	56	4	1.0
Test substance	(150 µg/plate)	+	58	61	48	56	7	1.0
Test substance	(500 µg/plate)	+	70	49	61	60	11	1.0
Test substance	(1500 µg/plate)	+	44	56	41	47	8	0.8
Test substance	(5000 µg/plate)	+	47	50	40	46	5	0.8
2-Nitrofluorene	(2 µg/plate)	-	349	467	412	409	59	8.9
Benzo[a]pyrene	(5 µg/plate)	+	641	560	627	609	43	10.5
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	164	196	153	171	22	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 12: Results obtained with *S. typhimurium* TA100: main mutation test 2

Tester strain: <i>S. typhimurium</i> TA100								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 2 (with pre-incubation)								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	180	158	169	169	11	-	
Test substance (50 µg/plate)	-	177	158	175	170	10	1.0	
Test substance (150 µg/plate)	-	159	160	169	163	6	1.0	
Test substance (500 µg/plate)	-	174	189	160	174	15	1.0	
Test substance (1500 µg/plate)	-	162	174	171	169	6	1.0	
Test substance (5000 µg/plate)	-	192	214	167	191	24	1.1	
Water (0.1 mL/plate)	+	213	177	194	195	18	-	
Test substance (50 µg/plate)	+	196	153	167	172	22	0.9	
Test substance (150 µg/plate)	+	196	216	212	208	11	1.1	
Test substance (500 µg/plate)	+	204	203	169	192	20	1.0	
Test substance (1500 µg/plate)	+	177	181	212	190	19	1.0	
Test substance (5000 µg/plate)	+	147	201	167	172	27	0.9	
Sodium azide (2 µg/plate)	-	1163	1142	1215	1173	38	6.9	
2-Aminoanthracene (5 µg/plate)	+	1437	1618	1830	1628	197	8.3	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	189	147	158	165	22	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

TABLE 13: Results obtained with *S. typhimurium* TA1535: main mutation test 2

Tester strain: <i>S. typhimurium</i> TA1535								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 2 (with pre-incubation)								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	30	26	27	28	2	-	
Test substance (50 µg/plate)	-	19	26	23	23	4	0.8	
Test substance (150 µg/plate)	-	19	23	28	23	5	0.8	
Test substance (500 µg/plate)	-	26	19	21	22	4	0.8	
Test substance (1500 µg/plate)	-	15	21	17	18	3	0.6	
Test substance (5000 µg/plate)	-	19	19	22	20	2	0.7	
Water (0.1 mL/plate)	+	28	22	23	24	3	-	
Test substance (50 µg/plate)	+	26	19	28	24	5	1.0	
Test substance (150 µg/plate)	+	26	27	24	26	2	1.1	
Test substance (500 µg/plate)	+	21	22	21	21	1	0.9	
Test substance (1500 µg/plate)	+	13	22	18	18	5	0.8	
Test substance (5000 µg/plate)	+	27	16	24	22	6	0.9	
Sodium azide (2 µg/plate)	-	1312	1342	1128	1261	116	45.0	
2-Aminoanthracene (5 µg/plate)	+	284	325	241	283	42	11.8	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	172	155	152	160	11	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

TABLE 14: Results obtained with *S. typhimurium* TA1537: main mutation test 2

Tester strain: <i>S. typhimurium</i> TA1537								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 2 (with pre-incubation)								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	15	10	16	14	3	-	
Test substance (50 µg/plate)	-	14	14	15	14	1	1.0	
Test substance (150 µg/plate)	-	14	13	17	15	2	1.1	
Test substance (500 µg/plate)	-	15	12	12	13	2	0.9	
Test substance (1500 µg/plate)	-	17	16	11	15	3	1.1	
Test substance (5000 µg/plate)	-	14	19	18	17	3	1.2	
Water (0.1 mL/plate)	+	39	38	41	39	2	-	
Test substance (50 µg/plate)	+	28	31	30	30	2	0.8	
Test substance (150 µg/plate)	+	38	42	45	42	4	1.1	
Test substance (500 µg/plate)	+	39	39	36	38	2	1.0	
Test substance (1500 µg/plate)	+	40	41	32	38	5	1.0	
Test substance (5000 µg/plate)	+	38	40	35	38	3	1.0	
9-Aminoacridine (50 µg/plate)	-	472	550	724	582	129	41.6	
Benzo[a]pyrene (5 µg/plate)	+	159	150	131	147	14	3.8	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	220	204	217	214	9	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

TABLE 15: Results obtained with *E. coli* WP2 *uvrA* (pKM101): main mutation test 2

Results obtained with <i>E. coli</i> WP2 <i>uvrA</i> (pKM101)								
Test substance: ABM-FD (Extract of Himematsutake)								
Test No.: Main test 2 (with pre-incubation)								
Addition	S9 mix + present - absent	Revertant colony counts/plate					Standard Deviation	Fold increase relative to vehicle
		Individual plate counts			Mean			
Water (0.1 mL/plate)	-	172	199	175	182	15	-	
Test substance (50 µg/plate)	-	143	128	145	139	9	0.8	
Test substance (150 µg/plate)	-	188	175	160	174	14	1.0	
Test substance (500 µg/plate)	-	181	159	186	175	14	1.0	
Test substance (1500 µg/plate)	-	126	141	155	141	15	0.8	
Test substance (5000 µg/plate)	-	162	112	158	144	28	0.8	
Water (0.1 mL/plate)	+	206	249	204	220	25	-	
Test substance (50 µg/plate)	+	228	169	235	211	36	1.0	
Test substance (150 µg/plate)	+	233	180	216	210	27	1.0	
Test substance (500 µg/plate)	+	225	238	189	217	25	1.0	
Test substance (1500 µg/plate)	+	160	182	198	180	19	0.8	
Test substance (5000 µg/plate)	+	216	201	180	199	18	0.9	
4-Nitroquinoline-1-oxide (2 µg/plate)	-	1173	986	1112	1090	95	6.0	
2-Aminoanthracene (10 µg/plate)	+	492	620	564	559	64	2.5	
Viable cell count: 10 ⁻⁶ dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	241	240	198	226	25	-
Sterility checks (total colony counts):								
Buffer	-	0	0	0	0	0	-	
S9 mix	+	0	0	0	0	0	-	
Test substance (5000 µg/plate)	-	0	0	0	0	0	-	

APPENDIX 1: Historical Control Data

Presented below are the historical control data from the period 1 December 1999 to 30 November 2004.

Water/Untreated

	TA100		TA1535		WP2 <i>uvrA</i> (pKM101)		TA98		TA1537	
	-	+	-	+	-	+	-	+	-	+
S9 Mix	-	+	-	+	-	+	-	+	-	+
Maximum	208	240	35	35	307	344	63	79	42	58
Minimum	82	89	7	8	65	80	21	30	7	8
Mean	138	151	20	20	132	162	38	48	16	25
No. of values	359	357	361	360	359	359	361	359	361	359
Standard deviation	23	27	4	4	30	35	6	8	6	9
Upper 99% limit	198	221	31	29	209	252	54	70	32	50
Lower 99% limit	78	80	8	10	56	71	22	27	0	1

Positive Controls

	TA100				TA1535			
	-	-	+	+	-	-	+	+
S9 Mix	-	-	+	+	-	-	+	+
	NaN ₃ (0.5 µg)	NaN ₃ (2 µg)	B[a]P (5 µg)	AAN (5 µg)	NaN ₃ (0.5 µg)	NaN ₃ (2 µg)	AAN (2 µg)	AAN (5 µg)
Maximum	1534	1240	1373	3204	1130	1256	1145	486
Minimum	208	478	212	589	49	732	40	136
Mean	579	845	731	1908	382	941	171	317
No. of values	985	36	996	37	980	29	991	29
Standard deviation	176	194	181	549	168	130	123	88

	WP2 <i>uvrA</i> (pKM101)				TA98		TA1537	
	-	-	+	-	-	+	-	+
S9 Mix	-	-	+	-	-	+	-	+
	AF-2 (0.05 µg)	NQO (2 µg)	AAN (10 µg)	2NF (1 µg)	2NF (2 µg)	B[a]P (5 µg)	AAC (50 µg)	B[a]P (5 µg)
Maximum	3749	3301	2305	4280	577	1070	3307	2115
Minimum	126	735	188	112	298	99	73	64
Mean	750	1658	766	434	435	575	891	271
No. of values	941	121	982	992	39	1044	507	1009
Standard deviation	425	493	301	461	74	179	480	104

NaN ₃	Sodium azide
AF-2	2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
2NF	2-Nitrofluorene
AAC	9-Aminoacridine
B[a]P	Benzo[a]pyrene
AAN	2-Aminoanthracene
NQO	4-Nitroquinoline-N-oxide

APPENDIX 2: GLP Compliance Statements



THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY

TEST TYPE

Huntingdon Life Sciences
Eye Research Centre
Occold
Eye
Suffolk
IP23 7PX

Analytical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Toxicology
Phys/Chem Tests

DATE OF INSPECTION

22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority



**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM**

GOOD LABORATORY PRACTICE

**STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC**

LABORATORY

TEST TYPE

**Huntingdon Life Sciences
Huntingdon Research Centre
Woolley Road
Alconbury
Huntingdon
Cambs.
PE28 4HS**

**Analytical Chemistry
Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Toxicology
Phys/Chem Tests**

DATE OF INSPECTION

07th April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority



**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM**

GOOD LABORATORY PRACTICE

**STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC**

LABORATORY	TEST TYPE
Huntingdon Life Sciences Eye Research Centre Occold Eye Suffolk IP23 7PX	Analytical Chemistry Clinical Chemistry Ecosystems Environmental Fate Environmental Toxicity Mutagenicity Toxicology Phys/Chem Testing

DATE OF INSPECTION

12th April 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

A handwritten signature in black ink, appearing to read 'Bryan J. Wright', with the date '12/05' written below it.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority



**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM**

GOOD LABORATORY PRACTICE

**STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC**

LABORATORY	TEST TYPE
Huntingdon Life Sciences Huntingdon Research Centre Woolley Road Alconbury Cambridgeshire PE28 4HS	Analytical Chemistry Clinical Chemistry Ecosystems Environmental Fate Environmental Toxicity Toxicology Phys/Chem Testing

DATE OF INSPECTION

7th March 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

A handwritten signature in black ink, reading 'Bryan J. Wright' with the date '9/6/05' written below it.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority

APPENDIX 3: MolTox™ S9 Quality Control Certificates

**MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9)
QUALITY CONTROL & PRODUCTION CERTIFICATE**

LOT NO.: <u>1797</u>	SPECIES: <u>Rat</u>	PREPARATION DATE: <u>December 2, 2004</u>
PART NO.: <u>11-101</u>	STRAIN: <u>Sprague Dawley</u>	EXPIRATION DATE: <u>December 2, 2006</u>
VOLUME: <u>1 ml & 2 ml</u>	SEX: <u>Male</u>	BUFFER: <u>0.154 M KCl</u>
	TISSUE: <u>Liver</u>	INDUCING AGENT(s): <u>Aroclor 1254</u>
REFERENCE: <u>Maron, D & Ames, B, <i>Mutat Res</i> 113:173, 1983</u>		<u>(Monsanto KL615), 500 mg/kg i.p.</u>
STORAGE: <u>At or below -70°C</u>		

BIOCHEMISTRY:**- PROTEIN**36.6 mg/mlAssayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.**- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES**

<u>Activity</u>	<u>P450</u>	<u>Fold - Induction</u>
EROD	IA1, IA2	140.2
PROD	2B1	33.7
BROD	2B1	40.3
MROD	1A2	105.0

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 46.3, 21.5, 77.4, & 10.4 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:**- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS**

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ CPA/	
<u>TA98</u> <u>TA1535</u>	
250.4	1678

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

µl S9 per plate/number his⁺ revertants per plate

<u>Promutagen</u>	<u>0</u>	<u>1</u>	<u>5</u>	<u>10</u>	<u>20</u>	<u>50</u>
BP (5 µg)	199	323	473	668	1104	1301
2-AA (2.5 µg)	194	1044	2219	2442	2611	2503

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**MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9)
QUALITY CONTROL & PRODUCTION CERTIFICATE**

LOT NO.: <u>1760</u>	SPECIES: <u>Rat</u>	PREPARATION DATE: <u>September 9, 2004</u>
PART NO.: <u>11-101</u>	STRAIN: <u>Sprague Dawley</u>	EXPIRATION DATE: <u>September 9, 2006</u>
VOLUME: <u>2 ml</u>	SEX: <u>Male</u>	BUFFER: <u>0.154 M KCl</u>
	TISSUE: <u>Liver</u>	INDUCING AGENT(s): <u>Aroclor 1254</u> (Monsanto KL615), 500 mg/kg i.p.
REFERENCE: <u>Maron, D & Ames, B, <i>Mutat Res</i> 113:173, 1983</u>		
STORAGE: <u>At or below -70°C</u>		

BIOCHEMISTRY:**- PROTEIN**

41.6 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.**- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES**

Activity	P450	Fold - Induction
EROD	IA1, IA2	143.4
PROD	2B1	22.8
BROD	2B1	17.0
MROD	1A2	77.7

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 41.3, 18.3, 40.0, & 14.3 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:**- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS**

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ CPA/	
<u>TA98</u> <u>TA1535</u>	
350.8 942	

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

µl S9 per plate/number his⁺ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 µg)	96	251	301	440	671	795
2-AA (2.5 µg)	101	984	1754	1651	1787	869

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**MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9)
QUALITY CONTROL & PRODUCTION CERTIFICATE**

LOT NO.: <u>1817</u>	SPECIES: <u>Rat</u>	PREPARATION DATE: <u>January 25, 2005</u>
PART NO.: <u>11-101</u>	STRAIN: <u>Sprague Dawley</u>	EXPIRATION DATE: <u>January 25, 2007</u>
VOLUME: <u>1 ml & 2 ml</u>	SEX: <u>Male</u>	BUFFER: <u>0.154 M KCl</u>
	TISSUE: <u>Liver</u>	INDUCING AGENT(s): <u>Aroclor 1254</u> <u>(Monsanto KL615), 500 mg/kg i.p.</u>
REFERENCE: <u>Maron, D & Ames, B, <i>Mutat Res</i> 113:173, 1983</u>		
STORAGE: <u>At or below -70°C</u>		

BIOCHEMISTRY:**- PROTEIN**

36.9 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.**- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES**

	Activity	P450	Fold - Induction
EROD	IA1, IA2		82.8
PROD	2B1		27.9
BROD	2B1		43.6
MROD	1A2		89.6

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 40.3, 16.2, 11.3, & 39.9 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:**- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS**

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ CPA/	
<u>TA98</u> <u>TA1535</u>	
193.6	1046

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

µl S9 per plate/number his⁺ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 µg)	102	175	542	904	1204	1463
2-AA (2.5 µg)	116	310	1875	2104	2280	2004

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APPENDIX 4: Formulation Chemistry

ABM-FD

FORMULATION CHEMISTRY

Authors:

Paul Mann,
David Fleet.

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INTRODUCTION

This report describes the analytical and sampling procedures used, and details the results obtained for the determination of concentrations of ABM-FD in test formulations analysed during the study.

The formulations for this study were prepared as solutions of ABM-FD (batch EX200408) in water by Genetic Toxicology personnel at Huntingdon Life Sciences Ltd.

The analytical work was undertaken by Formulation Chemistry and Central Laboratory Services (CLS) personnel at the Huntingdon Research Centre, Huntingdon Life Sciences between 10 January 2005 and 27 February 2005.

EXPERIMENTAL PROCEDURE

ANALYTICAL PROCEDURE

The analytical method^{Ref.2} was based on methodology supplied by the Sponsor^{Ref.1}. The method involved enzyme and acid hydrolysis of the test sample and determination of the resulting glucose content by CLS personnel at the Huntingdon Research Centre.

The analytical procedure was validated for ABM-FD in water formulations as part of an earlier study with respect to the specificity of the analysis, and method accuracy and precision at nominal concentrations of 50 mg/mL and 200 mg/mL. The experimental details and the analytical results obtained were presented in Huntingdon Life Sciences report IKI 001/052114.

CONCENTRATION IN TEST FORMULATIONS

Freshly prepared test formulations were sampled (25 ml) by Genetic Toxicology personnel at the Eye Research Centre and the samples submitted for analysis. On receipt, each treated sample was sub-sampled in duplicate (2 × 5 ml) and analysed in accordance with the analytical procedure.

The concentration of ABM-FD in aqueous formulations, analysed with respect to the glucose content, was conducted as part of an earlier study and confirmed at nominal concentrations of 50 mg/mL and 200 mg/mL during ambient temperature storage for 1 day and refrigerated storage for 8 days. The experimental details and analytical results obtained were presented in Huntingdon Life Sciences report IKI 001/052114.

Shared reagents were used in the analysis of dose formulations of this study and of Huntingdon Life Sciences study no. IKI/003.

REFERENCES

1. β -glucan analytical method, client e-mail of 25 June 2004.
2. Fleet D., Johnson, S.F., Mann, P., (2004). Analysis of ABM-FD in aqueous formulations. Huntingdon Life Sciences Analytical Method HRC/FCH/M80/04 Issue 01/011104.

RESULTS AND DISCUSSION

The mean concentrations of ABM-FD in test formulations, analysed with respect to the glucose content, and the deviation of mean results from nominal values are summarised in Table 1. The mean analysed concentrations were between 6% below and 12% above nominal values. The precision of duplicate analyses was <5.5%.

CONCLUSION

The mean concentrations of ABM-FD in test formulations, analysed with respect to the glucose content, were within -6% and +12% from the nominal concentrations.

TABLES

1. CONCENTRATIONS OF ABM-FD IN TEST FORMULATIONS

Date of formulation	Nominal inclusion (mg/ml)	Analysed concentration (mg/ml)			RME (%)
		Analysis 1	Analysis 2	Mean	
10-Jan-05	0	ND	-	ND	-
	50	49.6	44.4	47.0	-6.0
24-Jan-05	0	ND	-	ND	-
	50	49.1	48.4	48.7	-2.6
21-Feb-05	0	ND	-	ND	-
	50	53.9	58.2	56.0	12.0

RME Relative mean error, representing the deviation from nominal

ND None detected