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NOTOX
Safety & Environmental Research B.V.

REPORT

EVALUATION OF THE MUTAGENIC ACTIVITY OF

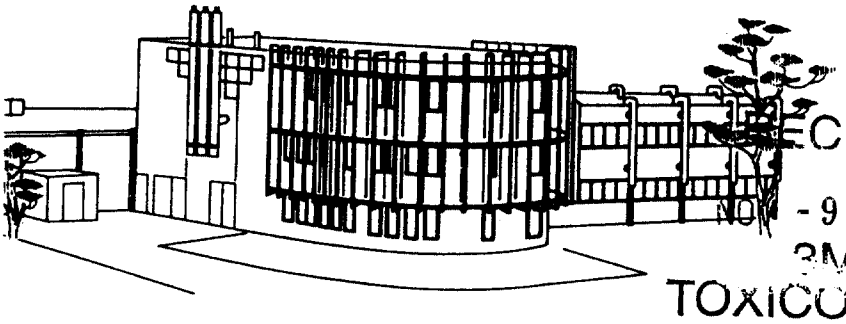
T-5874

IN AN IN VITRO MAMMALIAN CELL GENE MUTATION TEST

WITH L5178Y MOUSE LYMPHOMA CELLS

(WITH INDEPENDENT REPEAT)

NOTOX Project 115921
NOTOX Substance 38187



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T-5874,3

REPORT

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STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

With the exception that the stability of the test substance in the vehicle was unknown

Study Director Ing. E.J. van de Waart

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Date: 19/04/1994

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands.

Study procedures were subject to periodic inspections and general non study specific processes were also inspected at periodic intervals.

This report was audited by the NOTOX Quality Assurance Unit and the methods and results accurately reflect the raw data.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
January 17, 1994 February 10, 1994 February 23, 1994 March 28, 1994	January 17, 1994 February 14, 1994 February 23, 1994 March 28, 1994

Quality Assurance Manager C.J. Mitchell B.Sc.

Date: 20-4-94.

REPORT APPROVAL

STUDY DIRECTOR: Ing. E.J. van de Waart

.....

Date 19/04/1994

MANAGEMENT: Dr. I.C. Enninga
Technical Director

.....

Date 20/04/1994

PREFACE

Sponsor	3M Belgium - Chemical EBC Canadastraat 11 B-2070 ZWIJNDRECHT Belgium
Study Monitor	Mr. R.H. Cox
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. E.J. van de Waart
Technical Coordinator	C.M. Verspeek
Study Plan	Start : January 18, 1994 Completed : February 14, 1994

TEST SUBSTANCE

Identification	T-5874
Description	Cream solid
Batch	2334
Purity	100%
Specific Gravity	1.7
Instructions for test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	January 01, 1996
Stable for at least 4 hours in vehicle	Water : no Dimethylsulphoxide: not indicated

VEHICLE

The test substance was dissolved in dimethylsulphoxide of spectroscopic quality (Merck). Test substance concentrations were prepared directly prior to use. The final concentration of the solvent in the culture medium amounted to 0.8% (v/v).

GUIDELINES

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Guideline no. 476: "Genetic Toxicology: In Vitro Mammalian Cell Gene Mutation Tests", (adopted April 4, 1984).

- European Economic Community (EEC), Directive 87/302/EEC. Annex V of the EEC Directive 67/548/EEC, Part B: Methods for the Determination of Toxicity; "Other Effects-Mutagenicity: In Vitro Mammalian Cell Gene Mutation Test". EEC Publication no. L133 (adopted May 30, 1988).

ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test article reference sample, all specimens and raw data.

OBJECTIVE

Purpose of the study

The objective of this study was to evaluate the test substance for its ability to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells. The assay was conducted in the absence and presence of a metabolic system (S9-mix). The TK mutational system detected base pair mutations, frame shift mutations and small deletions.

Justification for selection of the test system

L5178Y mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes. The TK mutational system is able to detect base pair alterations, frame shift mutations and small deletions.
Cells deficient in thymidine kinase (TK), due to the forward mutation ($TK^{+}/-$ to $TK^{-}/-$) are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). TK deficient cells can not incorporate the analogue into its phosphorylated derivative (nucleotide); the nucleotides needed for cellular metabolism are obtained solely from de novo synthesis. In the presence of TK, TFT is converted into nucleotides, which are lethal to the cells. Thus, cells which will survive in culture medium containing TFT are mutated, either spontaneously or by the action of the test substance, giving rise to a TK deficient phenotype.
A test article which induces a positive response in this assay is presumed to be a potential mammalian cell mutagen.

MATERIALS AND METHODS

TEST SYSTEM

Test System	L5178Y mouse lymphoma cells
Rationale	Recognized by the international guidelines as the recommended test system (e.g. EPA, OECD, EEC).
Source	Dr. A.G.A.C. Knaap, Department of Radiation Genetics and Chemical Mutagenesis of the State University of Leiden, The Netherlands (1981). This mouse lymphoma cell line was originally derived from the Fischer L5178Y line, isolated by Clive (1975)*

*) Clive, D. and Spector, J.F.S., 1975, Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells, Mutation Res., 31, 17-29.

Stock cultures of these cells were stored in liquid nitrogen (-196°C). The cultures were checked for mycoplasma contamination.

CELL CULTURE

Cell culture conditions	L5178Y mouse lymphoma cells were cultured in F10 complete culture medium. Cultures were incubated in a humid atmosphere containing 5% CO_2 in air at 37°C. Cell density was preferably kept below 7×10^5 cells/ml. Cells were exposed to the test substance in F10 complete culture medium without serum, buffered with 20 mM HEPES.
F10 complete culture medium	F10 complete culture medium consisted of Ham's F10 medium without thymidine and hypoxanthine (Gibco), supplemented with 10% horse serum, L-glutamine (2 mM) and penicillin/streptomycin (50 U/ml and 50 µg/ml respectively).
Selective medium	Selective medium consisted of F10 complete culture medium which contained, in addition 0.33% Agar Noble (Difco) and 5 µg/ml TFT (Sigma).
Cloning medium	Cloning medium consisted of F10 complete culture medium, supplemented with 10% horse serum, which was transformed into a gel by addition of 0.33% Agar Noble.

Environmental conditions

All incubations were carried out in a humid atmosphere (80-95%) containing 5% CO_2 in air in the dark at 37°C. The temperature and CO_2-percentage were monitored during the experiment.

REFERENCE SUBSTANCES

Negative control:
The vehicle of the test article.

Positive controls:

Without metabolic activation (-S9-mix):
Ethylmethanesulphonate (EMS; CAS no. 62-50-0; purity 98%; Janssen Chimica) (2 mM) was used. EMS causes direct alkylation of DNA.

With metabolic activation (+S9-mix):
Dimethylnitrosamine (DMN; CAS-no. 62-75-9, purity 99%, Janssen Chimica) (0.5 mM) was used. DMN had to be activated by microsomal enzymes present in the S9-mix, resulting in a methyldiazonium ion which could react with cellular DNA.

Solvents for Reference Substances
Hank's balanced salt solution without calcium and magnesium.

Solutions of reference substances were prepared immediately before use.

METABOLIC ACTIVATION SYSTEM

Preparation of S9-homogenate*

Rat liver microsomal enzymes were routinely prepared from adult male Wistar or Sprague Dawley rats, which were obtained from BRL, Switzerland.

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the SOP's. The rats were injected intraperitoneally with a solution (20% w/v) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding sacrifice). The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C).

*) Ames, B.N., Mc Cann, J. and Yamasaki, E., 1975, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Res., 31, 347-364.

Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice during the test. S9-mix contained per ml: 1.02 mg $MgCl_2.6H_2O$; 2.46 mg KCl; 1.7 mg glucose-6-phosphate; 3.4 mg NADP; 4 µmol HEPES and 0.5 ml S9. The above solutions were mixed and filter (0.22 µm)-sterilized (apart from the S9-fraction, which was added after filter-sterilization of the S9-mix components). Metabolic activation was achieved by adding 0.2 ml liver S9-mix to each ml of cell suspension.

EXPERIMENTAL PROCEDURE

Selection of Dose Levels/Cytotoxicity Test

Prior to the actual mutagenicity test cytotoxicity data were obtained by treating 6×10^6 cells, suspended in 6 ml of F-10 medium buffered with 20 mM HEPES, in the absence of serum in a sterile 30 ml centrifuge tube with a range of test substance concentrations in approximately half log steps, both in the absence and presence of S9-mix. The centrifuge tubes were rotated for 3 h on a roller mixer at 37°C.
After the exposure, the cells were separated from the treatment solutions by 3 centrifugation steps (115 g, 8 min), each followed by removal of the supernatant and resuspension of the cells, twice in Hank's balanced salt solution and finally in F-10 medium. The cells in the final suspension were counted in an "Improved Neubauer" haemocytometer. Relative cytotoxicity, expressed as the reduction after approximately 24 h and 48 h growth compared to nontreated control cells, was used to determine a suitable concentration range (4 doses) of the test substance to be applied to cultures prepared for mutagenicity testing. In case the test substance was not toxic and/or difficult to dissolve in aqueous solutions the highest concentration was determined by the solubility in the culture medium. In general concentrations exceeding 5 mg/ml were not tested.

Cleansing

Prior to mutagenicity (and cytotoxicity) testing, the cells were grown for 1 day in culture medium containing 10^{-4} M hypoxanthine, 2×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT-medium) to reduce the amount of spontaneous mutants, followed by a recovery period of 2 days on medium containing hypoxanthine and thymidine only. After this period cells were returned to normal medium at least for 1 day before starting the experiment.

Mutagenicity test*

The test substance was tested both with and without S9-mix in two independent experiments. 6 x 10^6 cells (10^6/ml), or 12 x 10^6 cells (10^6/ml) for test substance concentrations expected to be strongly toxic, for each selected dose cells were exposed for 3 h to the test substance in HEPES-buffered Ham's F-10 medium without serum. For this purpose the cell suspensions were placed in 30 ml centrifuge tubes on a roller mixer at 37°C. Solvent and positive controls were included. After exposure, cells were washed twice with HBSS, counted and seeded in F-10 culture medium for expression of the mutant phenotype. The cultures were subcultured at least every other day in order to maintain log phase growth. The expression period for TFT-resistant mutants was 3 days. Immediately after exposure to the test substance 3 x 200 cells of each dose were plated into P90 petri dishes containing 15 ml cloning medium to determine cell survival (cloning efficiency). The cell survival was counted after 10-14 days with the Artek colony counter or the naked eye.

*) Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G. and Brown, M.M.M., 1979, Validation and characterization of the L5178Y/TK Mouse lymphoma mutagen assay system, Mutation Res., 59, 61-108.

*) Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A. and Salsburg, D.S., 1980, Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation, Mutation Res., 72, 447-474.

*) Jotz, M. and Mitchell, A.D., 1981, Effects of 20 coded chemicals on the forward mutation frequency at the thymidine kinase locus in L5178Y mouse lymphoma cells. In: Evaluation of short-term tests of carcinogens. F.J. de Serres and J. Ashby (Eds.), Elsevier-North Holland.

*) Van der Hoeven, J.C.M., Bruggeman, I.M. and Debets, F.M.H., 1984, Genotoxicity of quercetin in cultured mammalian cells, Mutation Res., 136, 9-21.

*) Clive, D., Caspary, W., Kirby, P.E., Krehl, R., Moore, M., Mayo, J. and Oberly, T.J., 1987, Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity, Mutation Res., 189, 143-156.

Mutant selection

After the expression period a total number of 1.5 x 10^6 cells were plated in ten P90 petri dishes, each containing 15 ml selective medium (TFT-selection). TK-deficient mutants formed microcolonies in 10-17 days, which were counted with the naked eye. Cloning efficiencies at the time of mutant selection were determined as described above.
The mutant frequency was expressed as the number of mutants per 10^5 surviving cells.

ACCEPTABILITY OF ASSAY

A mutation assay was considered acceptable if it met the following criteria:

a) The absolute cloning efficiency of the solvent controls was $\geq$ 50%.
b) At least three of the four doses of the test substance had an acceptable number of surviving cells (10^6) analysed for expression of the TK mutation.
c) The spontaneous mutant frequency in the untreated or solvent control was < 5 per 10^5 clonable cells.
d) The positive controls (ethylmethanesulfonate and dimethylnitrosamine) induced significant (at least 2-fold) increases in the mutant frequencies.

DATA EVALUATION AND STATISTICAL PROCEDURES

No formal hypothesis testing was done.
A test substance was considered positive (mutagenic) in the mutation assay if:

a) It induced at least a 2-fold increase in the mutant frequency compared to the solvent control in a dose-dependent manner; and
b) The results were reproducible in an independently repeated test.

A test substance was considered negative (not mutagenic) in the mutation assay if:

a) None of the tested concentrations showed a mutant frequency at least twice that of the solvent control.
b) The results were confirmed in an independently repeated test.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

RESULTS

CYTOTOXICITY TEST/DOSAGE SELECTION

Preliminary solubility tests indicated that T-5874 was soluble in DMSO at 120 mg/ml but precipitated in the exposition medium at concentrations of 333 µg/ml and upwards.
Table 1 shows the results of the preliminary cytotoxicity test with T-5874 in the presence and absence of a metabolic activation system (S9-mix). In the absence of S9-mix T-5874 inhibited the growth of the lymphoma cells in suspension at a test substance concentration of 100 µg/ml by 58% and at 333 µg/ml by 81%. In the presence of S9-mix the test substance inhibited the growth of the lymphoma cells in suspension at 333 µg/ml by 44%.

MUTAGENICITY TEST

Based on the results of the cytotoxicity test (Table 1), the following dose range was selected for mutagenicity testing:
Without S9-mix: 10 to 333 µg/ml culture medium
With S9-mix : 10 to 333 µg/ml culture medium

EMS (2 mM) was used as a positive control in the test without metabolic activation, whereas DMN, (0.5 mM) served as a positive control for the assay with metabolic activation. Tables 2 and 3 show the percentages of cell survival and the mutant frequencies for various concentrations of T-5874. Individual colony counts of cloning and selective plates, and cell counts during subculturing are listed in Tables 4-9 of the appendix.

In the absence of S9-mix the test substance induced a slight (2.3-fold) increase in the mutant frequency in experiment 1. In experiment 2 a slight, less than 2-fold increase in the mutant frequency was observed. However, the increase in the mutant frequency was not reproducible (not 2-fold in the second experiment) and not dose related. Therefore, the test substance is considered not mutagenic in the absence of S9-mix.
In the presence of S9-mix the test substance induced no significant increase in the mutant frequency in both independent experiments.

The spontaneous mutant frequencies in the solvent-treated control cultures were within our historical control data range (2.0 $\pm$ 1.2 in the absence of S9-mix and 1.4 $\pm$ 0.8 in the presence of S9-mix; indicated are means $\pm$ S.D. for n=40 and 41 respectively).
Mutant frequencies induced by positive control chemicals were increased by 5- to 7-fold for EMS and by 9- to 10-fold for DMN. It was therefore concluded that the test conditions were optimal and that the metabolic activation system (S9-mix) functioned properly.

CONCLUSION

In conclusion, T-5874 is not mutagenic in the TK mutation test system under the experimental conditions described in this report.

TABLE 1 PRELIMINARY CYTOTOXICITY DETERMINATION IN L5178Y MOUSE LYMPHOMA CELLS

Dose (µg/ml)	Cells/ml (x 10^5) After 0 h	After 24 h	After 48 h	Total	Suspension growth % of control
		Without metabolic activation (-S9-mix)			
Solvent control	5.9	6.3	4.5	65.3	100
1	6.2	5.1	4.7	58.1	89
3.3	6.1	4.9	5.4	63.0	96
10	4.8	4.2	4.4	34.7	53
33	4.9	4.8	5.1	46.9	72
100	5.2	3.1	4.4	27.7	42
333[3]	5.5	1.0[1]	3.7	12.7	19
		With metabolic activation (+S9 mix)			
Solvent control	6.5	6.0	6.4	97.5	100
1	6.4	6.0	6.7	100.5	103
3.3	6.4	6.6	6.1	100.7	103
10	6.0	5.9	6.1	84.4	87
33	6.3	7.4	5.9	107.4	110
100	6.2	6.2	5.7	85.6	88
333[3]	5.5	5.1	5.0	54.8	56

Solvent control = DMSO
Subculture = 1.6 x 10^5 cells/ml

[1] No subculture

[2] Total growth = Cell count after 0 h $\times \frac{\text{(24 h cells/ml)}}{\text{cells subcultured directly after treatment}} \times \frac{\text{(48 h cells/ml)}}{\text{cells subcultured after 24 h}}$

[3] Test substance precipitated slightly in the exposition medium

TABLE 2 CYTOTOXIC AND MUTAGENIC RESPONSE OF T-5874 IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

EXPERIMENT 1

DOSE (µg/ml)	C.E. AT DAY 0 (% OF CONTROL)	C.E. AT DAY 3 (ABSOLUTE %)	MEAN NO. OF MUTANTS PER PLATE	MUTATION FREQUENCY x 10^5
	Without metabolic activation (-S9-mix)			
Solvent control	100	60	2.1	2.3
10	102	61	2.4	2.6
33	103	60	3.4	3.8
100	90	62	4.8	5.2
333[1]	70	77	1.8	1.6
2 mM EMS	129	60	14.2	15.9
	With metabolic activation (+S9-mix)			
Solvent control	100	58	1.8	2.1
10	83	57	1.3	1.5
33	94	59	0.9	1.0
100	92	59	0.5	0.6
333[1]	61	83	2.6	2.1
0.5 mM DMN	30	38	11.3	19.8

C.E. = Cloning Efficiency
Solvent control = DMSO
EMS = Ethylmethanesulphonate
DMN = Dimethylnitrosamine
[1] Test substance precipitated slightly in the exposition medium

TABLE 3 CYTOTOXIC AND MUTAGENIC RESPONSE OF T-5874 IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

EXPERIMENT 2

DOSE (µg/ml)	C.E. AT DAY 0 (% OF CONTROL)	C.E. AT DAY 3 (ABSOLUTE %)	MEAN NO. OF MUTANTS PER PLATE	MUTATION FREQUENCY x 10^5
	Without metabolic activation (-S9-mix)			
Solvent control	100	85	4.2	3.3
10	95	98	5.8	4.0
33	106	99	3.7	2.5
100	59	94	6.1	4.3
333[1]	65	97	5.2	3.6
2 mM EMS	94	91	23.2	17.1
	With metabolic activation (+S9-mix)			
Solvent control	100	103	3.4	2.2
10	82	78	3.2	2.8
33	92	100	2.4	1.6
100	96	73	1.3	1.2
333[1]	91	101	3.9	2.6
0.5 mM DMN	41	46	14.9	21.6

C.E. = Cloning Efficiency
Solvent control = DMSO
EMS = Ethylmethanesulphonate
DMN = Dimethylnitrosamine
[1] Test substance precipitated slightly in the exposition medium

APPENDIX

Individual Colony Counts and Cell Counts during Expression Period

FORMULAS AND CALCULATIONS FOR L5178Y MOUSE LYMPHOMA TEST SYSTEM.

$$\text{Mutant frequency per } 10^5 \text{ survivors} = \frac{100}{\text{cloning efficiency}} \times \frac{\text{Total number of mutant colonies on selective plates}}{\text{number of seeded cells}}$$

$$\text{Cloning efficiency} = \frac{\text{Average No. of colonies on cloning plates}}{200} \times 100\%$$

L5178Y MOUSE LYMPHOMA TEST SYSTEM

- Cell counts during expression period
- Cloning efficiency immediately after exposure
- Mutation experiments, individual colony counts

Tables 4-6 Experiment 1
Tables 7-9 Experiment 2

Abbreviations used: DMN, dimethylnitrosamine
EMS, ethylmethanesulphonate
Solvent control: Dimethylsulphoxide

TABLE 4 L5178Y MOUSE LYMPHOMA TEST SYSTEM - CELL COUNTS AND SUBCULTURE DATA

Experiment 1

dose (µg/ml)	DAY 0				DAY 2			DAY 3	
	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	%[1]	Subculture x 10^6 Total amount[3]	Cell count c/ml x 10^5	%[2]	Subculture x 10^6 Total amount[4]	Cell count c/ml x 10^5	%[2]
Without Metabolic Activation (-S9-mix)									
DMSO	6	3.0	50	2.8	5.0	100	4.0	5.3	100
10	6	2.3	38	2.2	4.8	96	4.0	5.5	104
33	6	1.9	32	1.8	4.3	86	4.0	5.3	100
100	6	2.5	42	2.4	3.8	76	4.0	5.4	102
333	12	5.8	48	5.7	2.7	54	4.0	4.6	87
EMS	6	2.6	43	2.4	4.4	88	4.0	5.7	108
With Metabolic Activation (+S9-mix)									
DMSO	6	3.8	63	3.0	6.0	100	4.0	5.9	100
10	6	3.5	58	3.0	6.1	102	4.0	5.2	88
33	6	4.2	70	3.0	6.9	115	4.0	5.5	93
100	6	3.9	65	3.0	6.4	107	4.0	5.4	92
333	6	3.0	50	2.8	4.4	73	4.0	4.9	83
DMN	6	3.5	58	3.0	3.1	52	4.0	3.2	54

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of control}} \times 100\%$

(3) cell density 0.4×10^5 c/ml

(4) cell density 1.6×10^5 c/ml

TABLE 5 L5178Y MOUSE LYMPHOMA TEST SYSTEM - CLONING EFFICIENCY DAY 0

EXPERIMENT 1

Dose (µg/ml)	No. of colonies/ cloning plate 1	2	3	Mean No. of colonies/plate	Cloning efficiency absolute	relative (% of control)
				Without S9-mix		
Solvent control	109	105	108	107	54	100
10	111	113	104	109	55	102
33	106	112	112	110	55	103
100	91	101	97	96	48	90
333	98	68	60	75	38	70
EMS	137	146	131	138	69	129
				With S9-mix		
Solvent control	168	174	163	168	84	100
10	139	135	146	140	70	83
33	162	150	163	158	79	94
100	169	159	138	155	78	92
333	101	102	105	103	52	61
DMN	59	53	39	50	25	30

TABLE 6 L5178Y MOUSE LYMPHOMA TEST SYSTEM - SELECTION DATA AND CLONING EFFICIENCY

EXPERIMENT 1

Dose µg/ml	Number of colonies/Selection plate										Total No.	No. of colonies /cloning plate			Mean	MF
	1	2	3	4	5	6	7	8	9	10		1	2	3		
Without Metabolic Activation (-S9-mix)																
Solvent control	0	3	3	4	3	0	2	2	1	3	21	120	123	116	120	2.3
10	4	4	4	4	0	2	2	1	3	0	24	114	122	128	121	2.6
33	4	2	5	5	0	3	5	4	3	3	34	127	117	114	119	3.8
100	5	8	4	4	4	5	3	6	6	3	48	138	119	113	123	5.2
333	5	3	0	0	0	1	2	1	2	4	18	148	145	169	154	1.6
EMS	12	17	15	9	22	19	11	16	9	12	142	105	119	134	119	15.9
With Metabolic Activation (+S9-mix)																
Solvent control	1	0	1	3	2	4	1	3	2	1	18	118	117	114	116	2.1
10	0	1	2	2	1	0	2	2	1	2	13	110	115	117	114	1.5
33	0	1	1	1	0	0	3	0	0	3	9	114	119	117	117	1.0
100	0	1	0	0	0	0	2	0	0	2	5	112	117	125	118	0.6
333	1	1	3	5	1	1	3	1	5	5	26	165	183	151	166	2.1
DMN	20	13	11	10	10	7	12	11	11	8	113	75	73	81	76	19.8

MF = Mutant frequency per 10^5 survivors

TABLE 7 L5178Y MOUSE LYMPHOMA TEST SYSTEM - CELL COUNTS AND SUBCULTURE DATA

Experiment 2

dose (µg/ml)	DAY 0				DAY 2			DAY 3	
	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	%[1]	Subculture x 10^6 Total amount[3]	Cell count c/ml x 10^5	%[2]	Subculture x 10^6 Total amount[4]	Cell count c/ml x 10^5	%[2]
Without Metabolic Activation (-S9-mix)									
DMSO	6	4.1	68	3.0	4.2	100	4.0	6.0	100
10	6	4.3	72	3.0	3.0	71	4.0	6.1	102
33	6	3.9	65	3.0	3.2	76	4.0	4.8	80
100	6	4.3	72	3.0	2.1	50	4.0	6.0	100
333	12	9.0	75	6.0	2.0	48	4.0	5.8	97
EMS	6	4.0	67	3.0	4.4	105	4.0	6.0	100
With Metabolic Activation (+S9-mix)									
DMSO	6	3.7	62	3.0	5.4	100	4.0	6.3	100
10	6	4.4	73	3.0	5.1	94	4.0	5.2	83
33	6	3.9	65	3.0	5.4	100	4.0	6.2	98
100	6	3.8	63	3.0	5.9	109	4.0	4.5	71
333	6	3.5	58	3.0	4.4	81	4.0	5.9	94
DMN	6	4.0	67	3.0	2.0	37	4.0	3.7	59

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of control}} \times 100\%$

(3) cell density 0.4×10^5 c/ml

(4) cell density 1.6×10^5 c/ml

TABLE 8 L5178Y MOUSE LYMPHOMA TEST SYSTEM - CLONING EFFICIENCY DAY 0

EXPERIMENT 2

Dose (µg/ml)	No. of colonies/ cloning plate 1 2 3	Mean No. of colonies/plate	Cloning efficiency absolute	Cloning efficiency relative (% of control)
		Without S9-mix		
Solvent control	147 ; 147 ; 141	145	73	100
10	138 ; 134 ; 138	137	69	95
33	155 ; 147 ; 158	153	77	106
100	84 ; 85 ; INF	85	43	59
333	97 ; 92 ; 92	94	47	65
EMS	133 ; 135 ; 143	137	69	94
		With S9-mix		
Solvent control	168 ; 149 ; 189	169	85	100
10	130 ; 147 ; INF	139	70	82
33	167 ; 152 ; 149	156	78	92
100	161 ; 165 ; 162	163	82	96
333	166 ; 139 ; 156	154	77	91
DMN	75 ; 63 ; INF	69	35	41

INF = Plate infected with fungi

TABLE 9 L5178Y MOUSE LYMPHOMA TEST SYSTEM - SELECTION DATA AND CLONING EFFICIENCY

EXPERIMENT 2

Dose µg/ml	Number of colonies/Selection plate										Total No.	No. of colonies /cloning plate			Mean	MF
	1	2	3	4	5	6	7	8	9	10		1	2	3		
Without Metabolic Activation (-S9-mix)																
Solvent control	0	4	4	3	5	2	8	5	6	5	42	150	187	172	170	3.3
10	6	3	4	8	5	7	5	7	6	7	58	199	202	183	195	4.0
33	2	7	4	1	2	3	8	5	3	2	37	189	198	203	197	2.5
100	10	5	4	5	5	7	9	4	7	5	61	189	182	190	187	4.3
333	5	6	9	4	4	6	6	3	6	3	52	204	195	182	194	3.6
EMS	24	27	26	24	16	15	28	25	23	24	232	180	180	184	181	17.1
With Metabolic Activation (+S9-mix)																
Solvent control	3	3	3	4	4	2	4	3	4	4	34	201	226	190	206	2.2
10	3	3	4	8	1	5	4	2	2	0	32	144	151	171	155	2.8
33	4	0	1	1	3	4	6	3	2	0	24	201	204	194	200	1.6
100	1	0	2	0	1	6	1	0	2	0	13	161	140	138	146	1.2
333	3	6	7	5	2	1	4	3	5	3	39	180	223	201	201	2.6
DMN	12	15	12	18	17	16	18	13	12	16	149	77	105	95	92	21.6

MF = Mutant frequency per 10^5 survivors